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Biochemical Analysis of Cell Division Protein Complexes in Streptomyces coelicolor

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BIOCHEMICAL ANALYSIS OF CELL DIVISION PROTEIN COMPLEXES IN *STREPTOMYCES COELICOLOR*

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Allen M. Kotun

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BIOCHEMICAL ANALYSIS OF CELL DIVISION PROTEIN

COMPLEXES IN *STREPTOMYCES COELICOLOR*

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ABSTRACT

BIOCHEMICAL ANALYSIS OF CELL DIVISION PROTEIN COMPLEXES IN *STREPTOMYCES COELICOLOR*

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Thesis Supervised by Dr. Joseph R. McCormick

In *Streptomyces coelicolor*, at least six proteins are involved in the process of cell division: FtsZ, FtsQ, FtsL, DivIC, FtsW, and FtsI. The purpose of this study was to biochemically analyze putative cell division protein interactions to complement and possibly expand upon previous genetic studies. To accomplish this, *S. coelicolor* strains expressing epitope-tagged cell division proteins were created and pilot experiments using a Ni-NTA pull down assay were performed. His_8-FtsZ was found to be stable *in vivo*; however, many fusion proteins were found to be nonfunctional. The Ni-NTA pull down assay worked well to isolate His_8-FtsZ, but other fusion proteins could not be visualized by western blot analysis due to low levels of expression. The antibodies used in western blot analyses were found to be problematic due to high background and nonreactivity. Attempts to optimize these assays were performed; however, further optimization is needed to continue this study.

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INTRODUCTION

Cell Division in *Escherichia coli*

Cell division is a complex and essential process that allows unicellular bacteria to reproduce by duplicating their contents and dividing into two equal daughter cells. This process has been studied in a number of microorganisms, most notably *Escherichia coli* and *Bacillus subtilis*. In *E. coli*, at least fifteen proteins have been identified that are involved in this process: FtsZ, FtsA, ZipA, ZapA (also known as YgfE), FtsE, FtsX, FtsK, FtsQ, FtsB (previously named YgbQ), FtsL, FtsW, FtsI, FtsN, AmiC, and EnvC. Many of the genes that encode these proteins have been named filamentous temperature sensitive (*fts*) genes because they were first discovered while analyzing temperature-sensitive lethal mutants of *E. coli* that formed long filamentous cells when grown at high, nonpermissive temperatures (Hirota *et al*., 1968; Van De Putte *et al*., 1964). All of these proteins assemble at midcell during cell division and form a ring-like structure known as the divisome (Goehring and Beckwith, 2005; Vicente and Rico, 2006). In most bacteria, including *E. coli* and *B. subtilis*, many of these cell division proteins are required for the viability of the organism (Margolin, 2005). In *E. coli*, these proteins were believed to localize to the midcell in a strictly linear fashion where the localization of an early protein is essential for the localization of later proteins (Figure 1) (Buddelmeijer and Beckwith, 2002).

The first protein to localize at the future site of division is FtsZ (Bi *et al*., 1991). FtsZ is a 40 kDa cytoplasmic protein that is a prokaryotic homolog of tubulin (Löwe *et al*., 2004). The earliest known step of cell division is the formation of the FtsZ ring.

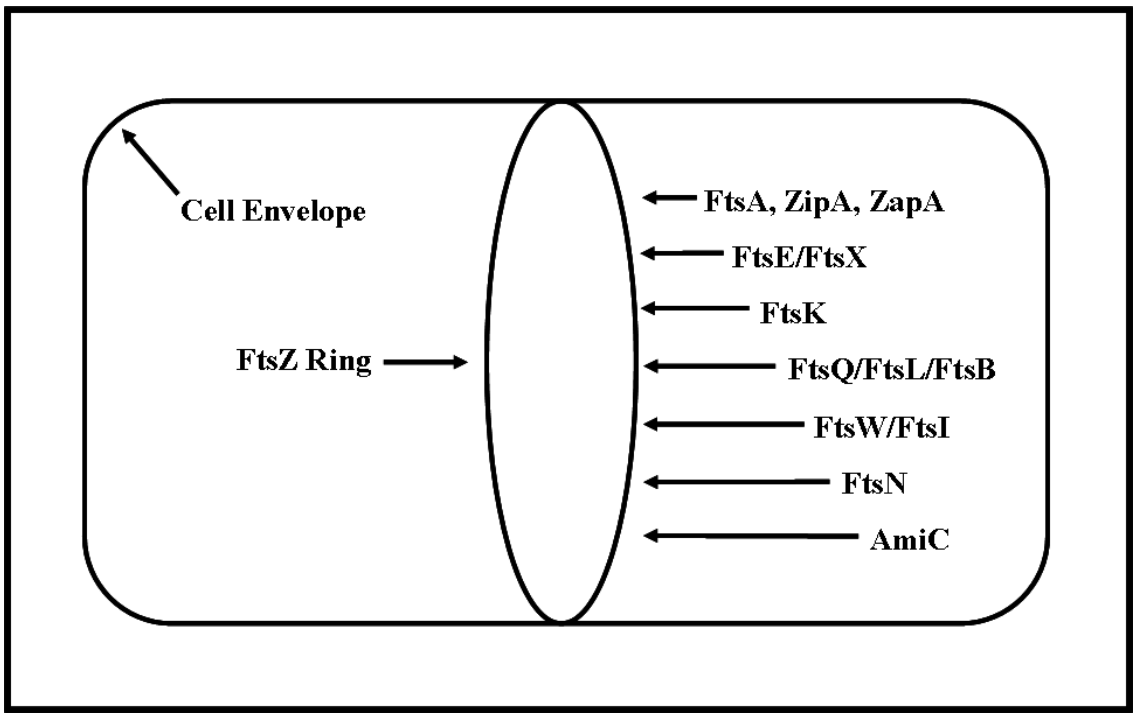

Figure 1

The order in which cell division proteins assemble in *E. coli* at the midcell position to form the divisome. FtsZ localizes first and forms the FtsZ ring. FtsA, ZipA, and possibly ZapA localize next, independently of each other. The remaining proteins localize as indicated (top to bottom), independently or as a complex of two or more proteins. The point at which EnvC (not pictured) localizes to the divisome has not yet been determined. (Adapted From: Weiss, 2004)

During this process, FtsZ localizes to the inner membrane of the cell envelope where it polymerizes in a GTP-dependent manner (de Boer *et al*., 1992). The FtsZ ring is believed to serve as scaffolding for the assembly of other cell division proteins and may provide the force needed for invagination of the cell wall during late cell division (Romberg and Levin, 2003). After division, FtsZ disassociates from the cell envelope and is once again found in the cytoplasm (Bi and Lutkenhaus, 1991; Addinall *et al*., 1996).

The next two proteins to localize to the midcell during cell division, FtsA and ZipA, are believed to be required for the stability of the FtsZ ring (Pichoff and Lutenhaus, 2002). FtsA is a peripheral membrane protein that contains an ATPase domain which is shared among members of the actin family of proteins (Sanchez *et al*., 1994; Bork *et al*., 1992). ZipA, or *ftsZ* interacting protein A, is a bitopic membrane protein believed to link the FtsZ ring to the cell envelope (Hale and de Boer, 1997). While FtsA is well conserved among many bacteria, ZipA has only been found in organisms closely related to *E. coli*. The localization of both proteins to the site of division is dependent on the prior localization of FtsZ (Addinall and Lutkenhaus, 1996; Hale and de Boer, 1999; Liu *et al*., 1999); and both proteins directly interact with a conserved motif located at the C-terminus of FtsZ (Liu *et al*., 1999; Ma and Margolin, 1999; Haney *et al*., 2001). When one protein is inactivated, FtsZ rings form and cell division can occur. Inactivating both proteins prevents FtsZ ring formation, however, which indicates that these proteins may have overlapping functions (Pichoff and Lutkenhaus, 2002). One important function these proteins share is the recruitment of other cell division proteins to the midcell (Hale and de Boer, 2002). FtsA is believed to be more important than ZipA, however, because certain mutations within *ftsA* have been found to completely bypass the need for ZipA, FtsK, and FtsN (Bernard *et al*., 2007; Geissler and Margolin, 2005; Geissler *et al*., 2003). Recent evidence suggests that FtsA may directly interact with FtsI and FtsN (Di Lallo *et al*., 2003; Corbin *et al*., 2004; Karimova *et al*., 2005).

ZapA is an FtsZ ring associated protein that is also believed to directly interact with FtsZ (Gueiros-Filho and Losick, 2002; Johnson *et al*., 2004). This small cytoplasmic

protein is nonessential for cell division in *E. coli* and is not required for the proper localization of other cell division proteins (Johnson *et al*., 2004; Goehring *et al*., 2005). ZapA is believed to exist as a dimer or tetramer in solution (Small *et al*., 2007). This protein has been found to bind polymers of FtsZ, to promote FtsZ polymer bundling, and to inhibit the GTPase activity of FtsZ (Small *et al*., 2007). ZapA fusions are currently being used as a novel way of detecting cell division protein interactions by prematurely targeting these proteins to the divisome (Goehring *et al*., 2005; Goehring *et al*., 2006).

The next proteins to localize to the divisome are FtsE and FtsX. FtsE and FtsX have homology to the ATP-binding cassette (ABC) transporter superfamily of proteins and are known to interact with one another to form a complex (Schmidt *et al*., 2004). FtsX is believed to interact with FtsA and FtsQ, while FtsE appears to directly interact with FtsZ (Karimova *et al*., 2005; Corbin *et al*., 2007). FtsEX requires FtsZ, FtsA, and ZipA for proper localization at the midcell and helps facilitate the localization of other cell division proteins, including FtsK (Schmidt *et al*., 2004). This protein complex is only required for the stability of FtsZ rings under conditions of low salt and low osmotic strength (Reddy, 2007).

The integral membrane protein FtsK localizes to the FtsZ ring after FtsEX. The later localization of FtsK is apparent in *ftsK*(Ts) mutants, which give rise to a highly indented filamentous phenotype at the nonpermissive temperature (Yu *et al*., 1998a). FtsK is a bifunctional protein. The N-terminus of FtsK is required for cell division in *E. coli*, while the C-terminus plays a role in DNA segregation (Draper and Gober, 2002; Yu *et al*., 1998b; Saleh *et al*., 2004; Pease *et al*., 2005). This C-terminal DNA translocase domain contains three subdomains: α, β, and γ (Yates *et al*., 2006). The DNA motor is

formed by the α and β subdomains, while the γ subdomain controls proper translocation of chromosomal DNA by interacting with DNA motifs called FtsK orienting polar sequences (KOPS) (Massey et al., 2006; Sivanathan et al., 2006).

Three bitopic proteins, FtsQ, FtsL, and FtsB, arrive at midcell after FtsK. These proteins are believed to form a complex independent of their localization to the divisome (Buddelmeijer and Beckwith, 2004). FtsQ is a 31 kDa protein that is essential for cell division in *E. coli* (Begg *et al*., 1980). This protein relies on FtsZ, FtsA, ZipA, and FtsK for proper localization to the FtsZ ring and is needed to recruit FtsL, FtsB, FtsI, and FtsN (Chen *et al*., 1999; Chen and Beckwith, 2001). FtsQ is also able to backrecruit FtsK under conditions when FtsK cannot localize to midcell, suggesting that these two proteins may interact directly (Goehring *et al*., 2005). Three functional subdomains of FtsQ have recently been identified that are believed to be involved in these interactions with other division proteins (D'Ulisse *et al*., 2007). FtsL and FtsB are both required for cell division. These two proteins are not only dependent on FtsQ and other earlier proteins in the pathway, they are also dependent on one another for proper localization at midcell (Buddelmeijer *et al*., 2002). In fact, FtsL is destabilized in the absence of FtsB (Buddelmeijer *et al*., 2002). Both proteins contain leucine zipper-like sequences in their periplasmic domains, which appear to be required for their interaction with one another (Ghigo and Beckwith, 2000; Buddelmeijer and Beckwith, 2004).

FtsW and FtsI, also known as penicillin-binding protein 3 (PBP3), are the next proteins to localize to the FtsZ ring. These proteins are believed to form a complex independent of their localization to midcell (Goehring et al., 2006). FtsW is an integral membrane protein that is essential for cell division and is required for the localization of

FtsI (Boyle *et al*., 1997; Pastoret *et al*., 2004). This protein appears to work in coordination with FtsI to catalyze septal peptidoglycan polymerization during cell division, however the mechanism of action is not yet known. FtsI is one of seven PBP's found in *E. coli* and is the only one that is essential for cell division (Spratt, 1977). This is one of the few cell division proteins with a known function. FtsI is a transpeptidase involved in septum-specific cell wall biosynthesis (Spratt, 1977; Pisabarro *et al*., 1986).

The last two proteins to join the divisome are FtsN and AmiC. FtsN is a bitopic protein that is essential for cell division in *E. coli* (Dai et al., 1993). Proper localization of this protein requires the prior localization of FtsZ and FtsA and also requires functional FtsI and FtsQ (Addinall *et al*., 1997). The C-terminus of FtsN is required for proper protein function, as well as localization to the midcell (Addinall *et al*., 1997). This gene was discovered as a multicopy suppressor of temperature-sensitive mutants of *ftsA*, *ftsQ*, and *ftsI* (Dai et al., 1993). Overexpression of FtsN was able to rescue cells depleted for FtsK (Draper *et al*., 1998). The N-terminus of the FtsN is required for suppressing the *ftsK* mutant phenotype, which may suggest that this domain is necessary for protein interactions and stabilizing the divisome (Goehring *et al*., 2007). AmiC requires FtsN for localization to midcell and is an amidase involved in daughter cell separation (Bernhardt and de Boer, 2003). This protein is nonessential for growth, as there are other amidases with overlapping function. However, AmiC appears to be the principle septum-cleaving enzyme, because mutants lacking this protein have the most severe cell separation phenotype when compared to other amidase mutants (Heidrich *et al*., 2001).

The most recent addition to the list of cell division genes is *envC*. This gene was discovered decades ago, but has not been well characterized until recently (Rodolakis *et al*., 1973; Bernhardt and de Boer, 2004). This recent study has shown that EnvC localizes to the midcell position during division, although the point at which EnvC localizes is not known at this time (Bernhardt and de Boer, 2004). This protein may have murein hydrolase activity, which could allow it to play a role in daughter cell separation (Bernhardt and de Boer, 2004). This gene is dispensable for growth, but is important for proper septation and separation of daughter cells (Hara *et al*., 2002).

Cell Division in *B. subtilis*

Cell division in *B. subtilis* is very similar to division in *E. coli*. With the exceptions of *zipA*, *ftsN*, and *envC*, homologs for all of the *E. coli* cell division genes have been found in this organism (Errington *et al*., 2003). The homologs for *ftsK*, *ftsQ*, *ftsB*, and *ftsI* are named *spoIIIE*, *divIB*, *divIC*, and *pbpB* (encodes the protein PBP2B), respectively. Two additional genes, *ezrA* and *sepF* (previously named *ylmF*), are also required for efficient cell division in *B. subtilis* (Levin *et al*., 1999; Hamoen *et al*., 2006; Ishikawa *et al*., 2006).

EzrA is a transmembrane protein that directly interacts with FtsZ and requires FtsZ to localize to midcell during division (Levin *et al*., 1999; Haeusser et al., 2004). EzrA was first identified in *B. subtilis* as a negative regulator of FtsZ assembly because the loss of this protein was found to lower the amount of FtsZ needed to form stable FtsZ rings, leading to the formation of additional FtsZ rings and septa (Levin *et al*., 1999). This protein is evenly distributed throughout the plasma membrane in both nondividing

and dividing cells, where it is believed to inhibit FtsZ polymerization by decreasing the binding affinity of GTP to FtsZ (Levin *et al*., 1999; Chung *et al*., 2007). This protein has also been found to act as a positive regulator of cell division, because when EzrA is expressed at low levels cell division is partially inhibited resulting in a filamentous phenotype (Chung *et al*., 2004). Because EzrA shares similar topology with ZipA and because both proteins interact with FtsZ, it is believed that these two proteins could be functionally homologous (Errington *et al*., 2003).

SepF, a protein involved in septum development, interacts with FtsZ and requires FtsZ to localize to midcell (Hamoen *et al*., 2006; Ishikawa *et al*., 2006). This protein is conserved in Gram-positive bacteria, but is not required for viability (Hamoen *et al*., 2006). Deleting this gene simultaneously with either *ezrA* or *ftsA*, however, results in a synthetic lethal mutation, which may indicate overlapping roles (Hamoen *et al*., 2006; Ishikawa *et al*., 2006). This protein may act in a later stage of division because *sepF* deletion mutants display a cell division defect in which septa are formed slowly and aberrantly (Hamoen *et al*., 2006). Localization of this protein is not dependent on any late cell division proteins (Hamoen *et al*., 2006).

Another difference between cell division in *E. coli* and *B. subtilis* is the way in which proteins localize to midcell. While the cell division proteins of *E. coli* localize in what appears to be a linear fashion, many of the cell division proteins of *B. subtilis* (DivIB, DivIC, FtsL, PBP2B, and possibly FtsW) display interdependency with one another (Errington *et al*., 2003). Depleting or altering any of these proteins prevents the localization of the others to midcell (Errington *et al*., 2003). This interdependency found

in *B. subtilis* has provided insight into putative cell division protein interactions that have been further studied in both *E. coli* and *B. subtilis*.

Techniques Used to Study Cell Division Protein Interactions

The apparent linear order in which proteins assemble to form the divisome in *E. coli* was determined mainly by fluorescent microscopy studies (Buddelmeijer and Beckwith, 2002). Recent studies, however, have found many cell division protein interactions that indicate this pathway may be more complex. One technique used to discover these interactions is the bacterial two-hybrid system. This method relies on the interaction-mediated reconstitution of a transcriptional regulatory protein, which causes the expression of a reporter gene. Thus far, 29 putative cell division protein interactions have been uncovered utilizing this method in *E. coli* (Table 1) (Di Lallo *et al*., 2003; Karimova *et al*., 2005). Many interactions between DivIB, DivIC, FtsL, and PBP2B have also been found in *B. subtilis* utilizing this same method (Daniel *et al*., 2006). Based on the nature of this method, however, these results must be viewed with caution. One reason is that the bacterial two-hybrid system relies on overexpression of the two-hybrid fusion proteins. This could lead to false positives from potentially weak interactions. Another reason is because these analyses are performed *in vivo* allowing fusion proteins to be incorporated into the divisome, which could lead to additional false positives due to close proximity instead of a direct interaction. Lastly, a third protein being expressed *in vivo* could act as a bridging molecule between two cell division proteins allowing indirect interactions to be considered direct interactions.

Table 1

Cell Division Protein Interactions Detected by the Bacterial Two-Hybrid System

	FtsZ	FtsA	ZipA	FtsX	FtsK	FtsQ	FtsL	FtsB	FtsW	FtsI	FtsN
FtsZ	o	o	o		×						
FtsA		o		×		o				o	o
ZipA			×								
FtsX						×					
FtsK						**o**	×			×	
FtsQ						o	**o**	**o**	**o**	**o**	**o**
FtsL								**o**	×	×	
FtsB										×	
FtsW										o	×
FtsI										×	o
FtsN											×

× - indicates a positive interaction between two proteins as determined by bacterial-two hybrid analyses only
o - indicates protein-protein interactions studied by bacterial-two hybrid analyses as well as other genetic analyses
o - Indicates protein-protein interactions studied by bacterial-two hybrid analyses as well as other genetic and biochemical analyses
(Adapted From: Di Lallo *et al*., 2003 and Karimova *et al*., 2005)

Another novel approach for studying cell division protein interactions in *E. coli* is to prematurely target cell division proteins to midcell using a ZapA fusion (Goehring *et al*., 2005; Goehring *et al*., 2006). These studies have revealed that three interacting subcomplexes may actually come together to form the divisome: FtsZ-FtsA-ZipA, FtsQ-FtsL-FtsB, and FtsW-FtsI. It was found that the two latter subcomplexes directly interact with one another. These studies provide a different model for divisome assembly, one that more closely mimics the interdependency found among cell division

proteins in *B. subtilis* (Figure 2). If this model is true, the linear order of assembly previously described might actually only represent the temporal order in which cell division proteins localize, not a strictly linear process where each protein can only interact with a previously localized protein.

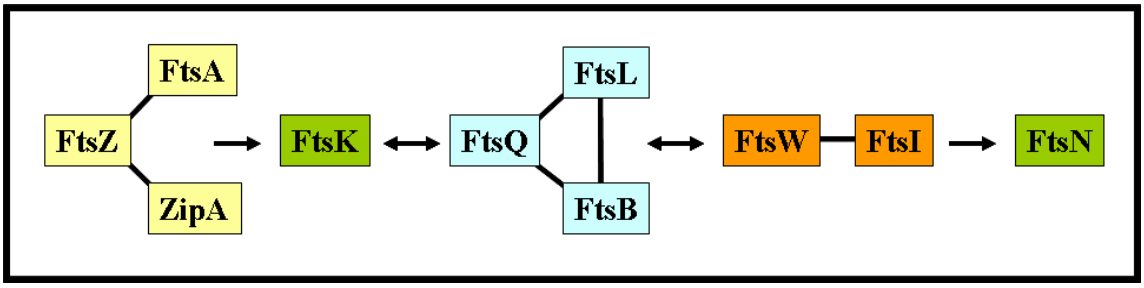

Figure 2

A new model for divisome organization in *E. coli*. The proteins in yellow represent the FtsZ ring complex. These proteins have previously been found to directly interact. The FtsQ-FtsL-FtsB (blue proteins) and the FtsW-FtsI (orange proteins) complexes have also been previously described. These complexes were identified in the premature targeting assay as interacting independently of FtsA. Double arrows represent other protein interactions identified in the premature targeting assay as FtsA independent. The requirements for FtsK and FtsN (green proteins) recruitment are unknown. The nonessential proteins ZapA, FtsE, FtsX, AmiC, and EnvC were not analyzed in the study used to create this model and are therefore not pictured.
(Adapted From: Goehring *et al*., 2006)

The last major method used to study cell division protein complexes is co-immunoprecipitation. In *E. coli*, this technique has lead to the discovery of the FtsQ-FtsL-FtsB complex and has been used to study an interaction between FtsZ and FtsE (Buddelmeijer and Beckwith, 2004; Corbin *et al*., 2007). This technique has also been used in *B. subtilis* to study the FtsZ-interacting proteins FtsA and SepF (Jensen *et al*., 2005; Ishikawa *et al*., 2006). While this method seems to be the most logical approach for studying true protein-protein interactions, it is not without its own problems. Most of

the proteins being studied are overexpressed in these experiments in order to visualize them via western blot analysis. This again could lead to weak interactions between proteins causing false positives.

Each of these methods used to study cell division protein interactions have been useful in discovering how the divisome is organized. Still, many of these putative complexes need to be verified by additional techniques. Because many of the cell division proteins are necessary for growth and viability, it can be difficult to genetically alter or delete the genes that code for them. Therefore, these types of experiments can be extremely complicated to perform in *E. coli* as well as other organisms.

Cell Division in *Streptomyces coelicolor*

Most studies pertaining to cell division genes have taken place in *E. coli* and *B. subtilis*, but this process is also studied in other organisms such as *Caulobacter crescentus* and *Streptomyces coelicolor* (Martin *et al*., 2004; Wang *et al*., 2001, Quardokus *et al*., 2001; McCormick and Losick, 1996; McCormick *et al*., 1994). The prokaryotic organism *S. coelicolor* is a Gram-positive soil bacterium that differs from other bacteria with respect to cell division. This organism grows by tip extension to form a mycelium, and, therefore, more closely resembles filamentous fungi than typical bacteria with regards to lifecycle. *S. coelicolor* produces vegetative filaments during vegetative growth and reproductive aerial filaments, which are necessary for sporulation, as the mycelium of the organism matures. The multinucleoid aerial filaments eventually synchronously produce cross-walls to create regularly spaced uninucleoid cells that eventually mature into spores (Chater, 1993).

S. coelicolor is an advantageous system for studying cell division for a number of reasons, most notably because *S. coelicolor* cell division mutants were found to remain viable. The first cell division gene homolog found in *S. coelicolor* was *ftsZ* (McCormick *et al*., 1994). This gene was found to be essential for dividing the aerial hyphae into chains of uninucleoid spores, but not for viability (McCormick *et al*., 1994). The *ftsZ*-null mutant is able to produce aerial hyphae, but unable to produce spores (McCormick *et al*., 1994). Similar studies have shown that additional mutants deleted for *ftsK*, *ftsQ*, *ftsL*, *divIC* (homolog of *E. coli ftsB*), *ftsW*, and *ftsI* are also viable (McCormick and Losick, 1996; Bennett, 2007; McCormick Laboratory, unpublished data).

The cell division mutants of *S. coelicolor* have been found to have interesting phenotypes. When the *ftsZ*- and *ftsQ*-null mutants are plated on minimal glucose agar, they over-produce a blue-pigmented antibiotic, actinorhodin, which forms a "blue halo" around individual colonies. Another interesting phenotype was found in the *ftsL*-, *divIC*-, *ftsW*-, and *ftsI*-null mutants. These cell division mutants have a rich medium-dependent prevention of sporulation (Bennett, 2007). All of these phenotypes can be used as a screen for identifying cell division mutants.

Another advantage to working with *S. coelicolor* is that the organism is genetically well characterized. A detailed physical map of the entire 9.7 Mb chromosome has been created and the genome has been sequenced (Redenbach *et al*., 1996; Bentley *et al*., 2002; Weaver *et al*., 2004). An ordered overlapping cosmid library for the chromosome has been constructed (Redenbach *et al*., 1996). A PCR targeting system for mutagenization of the chromosome has been adapted from *E. coli*, which greatly aids in the process of genetically altering this organism (Gust *et al*., 2002).

The purpose of this thesis was to biochemically analyze putative cell division protein interactions in *S. coelicolor*, an organism where the cell division genes are not required for viability, to complement and possibly expand upon previous genetic analyses (McCormick *et al*., 1994; McCormick and Losick, 1996; Bennett, 2007; McCormick Laboratory, unpublished data). The proteins FtsZ, FtsQ, FtsW, and FtsI were analyzed in this study to determine if cell division protein subcomplexes could be found in *S. coelicolor* and to possibly find additional interacting proteins. First, constructs containing epitope-tagged versions of these genes were created in *E. coli* using PCR-targeted mutagenesis. Many of the constructs necessary for this study were previously created (McCormick JR, unpublished data). These constructs were made mobilizable and introduced into the chromosome of the wildtype *S. coelicolor* strain M145 via homologous recombination after conjugation. Isolated strains were studied by phase-contrast microscopy and western blot analyses to determine if the epitope-tagged proteins behaved as wildtype and were stable. Lastly, a nickel-nitrilotriacetic acid (Ni-NTA) pull down assay was utilized to study possible cell division protein complexes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Cosmids

All *E. coli* and *S. coelicolor* strains used in this study are listed in Tables 2 and 3, respectively. Plasmids and cosmids used in this study are listed in Table 4.

Table 2

***E. coli* Strains Used in This Study**

Bacterial Strain	Genotype	Source or Reference
BL21(DE3)/pLysS	F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* (DE3) pLysS (cat^R)	Invitrogen
BT340	DH5α/pCP20	Datsenko and Wanner, 2000
BW25113	$lacI^q$ *rrnB* Δ*lacZ hsdR514* Δ*araBAD* Δ*rhaBAD*	Datsenko and Wanner, 2000
ET12567	*dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj201*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44* F^-	MacNeil *et al*., 1992

Table 3

***S. coelicolor* strains used in this study**

Bacterial Strain	Genotype[a]	Source or Reference
M145	Prototrophic $SCP1^-$ $SCP2^-$	Hopwood *et al*., 1985
HU133	Δ*ftsZ*::*aphI*	McCormick *et al*., 1994
HU151	Δ*ftsQ*::*aadA*	McCormick and Losick, 1996
JBY5	Δ*ftsI*::*aphI*	Yarnall, 2001
PFB26	Δ*ftsW*	Bidey, 2004

a) *aphI* - neomycin-resistance gene; *aadA* - spectinomycin-resistance gene

Table 4

General Plasmids and Cosmids Used in This Study

Plasmid/Cosmid	Description[a]	Source or Reference
C69	Cosmid containing the entire *S. coelicolor* division and cell wall gene cluster (amp^R, kan^R)	Redenbach *et al*., 1996
pCP20	FLP recombinase-expressing plasmid *rep101*^{ts} (cat^R)	Cherepanov and Wackernagel, 1995
pIJ773	pBluescript II SK(+) containing *aac(3)IV-oriT* disruption cassette flanked by *frt* sites (amp^R)	Gust *et al*., 2003
pIJ790	λ-RED (*gam*, *bet*, *exo*) *araC* *rep101*^{ts} (cat^R)	Gust *et al*., 2003
pIJ799	pBluescript II SK(+) containing *aac(3)IV-oriT* disruption cassette flanked by *bla* homology sites (amp^R)	Gust *et al*., 2003
pJY1	Expression vector used to overexpress His-tagged `FtsI	Yarnall, 2001
pUZ8002	RK2 derivative which is a nontransmissible helper plasmid in *E. coli* (kan^R)	Kieser *et al*., 2000

a) *aac(3)IV* - apramycin-resistance gene; *oriT* - origin of transfer site; *frt* - FLP recombinase target site; *bla* - ampicillin-resistance gene

Media and Growth Conditions

E. coli strains were grown on LB agar and in either LB, SOB, or 2X YT liquid media (Sambrook *et al*., 1989). All media were supplemented with the following antibiotics when appropriate: apramycin (50 μg/ml), ampicillin (100 μg/ml), nalidixic acid (30 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), and carbenicillin (100 μg/ml). All strains of *E. coli* were grown at 37°C, except the strains BT340, which contains a temperature sensitive plasmid and repressor, and BW25113/pIJ790, which

contains a temperature sensitive plasmid. These strains were grown at 30°C. The strain BT340 was grown at 42°C when induction of the FLP recombinase gene was necessary.

S. coelicolor strains were grown in YEME liquid medium or on R2YE, minimal glucose (MM), or soy flour mannitol (SFM) agar (Hopwood *et al*., 1985; Kieser *et al*., 2000). All media were supplemented with the following antibiotics when appropriate: spectinomycin (200 μg/ml), apramycin (25 μg/ml), nalidixic acid (20 μg/ml), kanamycin (160 μg/ml), and neomycin (10 μg/ml). All *S. coelicolor* strains were grown at 30°C.

DNA Techniques

Standard techniques were used in the extraction and purification of plasmid DNA, the creation of electrocompetent cells, and for transformation (Sambrook *et al*., 1989; Gust *et al*., 2002). *S. coelicolor* chromosomal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). Restriction enzymes were used according to manufacture's recommendations (New England Biolabs). Restriction enzyme-digested DNA fragments were excised from an agarose gel and purified, when necessary, by using the QIAquick Gel Extraction Kit (Qiagen). PCR products were purified, when necessary, by using the QIAquick PCR Purification Kit (Qiagen).

Creating Cosmids that Contain Epitope Tag Sequences within Cell Division Genes

All of the cosmid derivates discussed in this section were previously created (McCormick JR, unpublished data). The *in vivo* incorporation of the epitope tag sequences into the cell division genes was carried out by homologous recombination using a disruption cassette from the plasmid pIJ773. This disruption cassette contains

two FLP recombinase target (*frt*) sites surrounding an apramycin-resistance gene (*aac(3)IV*) and an origin of transfer (*oriT*) site. This 1382 bp cassette was removed from pIJ773 by digesting the plasmid with *Eco*RI and *Hin*dIII and gel purifying the fragment. The epitope tag-encoding sequences and homology extensions specific to either the 5' or 3' end of a specific cell division gene were added to the disruption cassette via PCR. Primers used to add epitope and homology sequences to the pIJ773 disruption cassette are listed in Tables 5 and 6.

Table 5

Primers Used to Create pIJ773 Disruption Cassettes that will Epitope Tag the 5' End of Cell Division Genes

Primer	Description[a]	Sequence (5' → 3')
oHA47	Universal primer used to add the *ha* epitope sequence to the 5' end of the disruption cassette	GGTCGACGGATCCCCGGAATGG CGTAGTCCGGGACGTCGTAGGG GTA
oHIS44	Universal primer used to add the his_8 epitope sequence to the 5' end of the disruption cassette	GGTCGACGGATCCCCGGAATGT GGTGGTGGTGGTGGTGGTGGTG
oW60HA	Used with oHA47 to add the *ha* epitope sequence and *ftsW* homology to the disruption cassette	GTGGGACGGTCCATGCCGACGT ACAGCGGAGGCCGCGATGTACC CCTACGACGTCCCGGA
oW64HIS	Used with oHIS44 to add the his_8 epitope sequence and *ftsW* homology to the disruption cassette	GTGGGACGGTCCATGCCGACGT ACAGCGGAGGCCGCGATGCACC ACCACCACCACCACCACCAC
oW59	Used with oHA47/oW60HA or oHIS44/ oW64HIS to add an additional site of *ftsW* homology to the disruption cassette	GCACGGGCGGACGTCCGGTACG GCTCTGGGGACTACCGGGTGTA GGCTGGAGCTGCT
oQ60HA	Used with oHA47 to add the *ha* epitope sequence and *ftsQ* homology to the disruption cassette	GCGCGCCGCTAGACACGTCTGA CGGAAGGCAGGGAGCGTGTACC CCTACGACGTCCCGGA
oQ64HIS	Used with oHIS44 to add the his_8 epitope sequence and *ftsQ* homology to the disruption cassette	GCGCGCCGCTAGACACGTCTGA CGGAAGGCAGGGAGCGTGCACC ACCACCACCACCACCACCAC

Table 5 (continued)

Primer	Description[a]	Sequence (5' → 3')
oQ59	Used with oHA47/oQ60HA or oHIS44/oQ64HIS to add an additional site of *ftsQ* homology to the disruption cassette	CCTGCTGGCGTGCACCGCGCTC GGCGGTGGTCGGTCCGGCTGTA GGCTGGAGCTGCTTC
oI60HA	Used with oHA47 to add the *ha* epitope sequence and *ftsI* homology to the disruption cassette	CGCCCACCCCGACCCCGACTCC CGGCAGGTGACGGAAGTGTACC CCTACGACGTCCCGGA
oI64HIS	Used with oHIS44 to add the his_8 epitope sequence and *ftsI* homology to the disruption cassette	CGCCCACCCCGACCCCGACTCC CGGCAGGTGACGGAAGTGCACC ACCACCACCACCACCACCAC
oI59	Used with oHA47/oI60HA or oHIS44/oI64HIS to add an additional site of *ftsI* homology to the disruption cassette	CGGGTCCGGGCACCCGGCGGCG CGGCGGTTCCCTGTCGGATGTA GGCTGGAGCTGCTTC
oZ64HIS	Used with oHIS44 to add the his_8 epitope sequence and *ftsZ* homology to the disruption cassette	CCGGGCGACACGTAACTCGAGG CGAGAGGCCTTCGACGTGCACC ACCACCACCACCACCACCAC
oZ59	Used with oHIS44/oZ64HIS to add an additional site of *ftsZ* homology to the disruption cassette	CGATGACTTTGATGACTGCGAG GTAGTTCTGCGGTGCTGCTGTA GGCTGGAGCTGCTTC

a) *ha* - hemagglutinin epitope tag sequence; his_8 - histidine-eight epitope tag sequence

Table 6

Primers Used to Create pIJ773 Disruption Cassettes that will Epitope Tag the 3' End of Cell Division Genes

Primer	Description	Sequence (5' → 3')
o3HA46	Universal primer used to add the *ha* epitope sequence to the 3' end of the disruption cassette	GAAGCAGCTCCAGCCTACATA CCCCTACGACGTCCCGGACTA CGCC
o3HIS43	Universal primer used to add the his_8 epitope sequence to the 3' end of the disruption cassette	GAAGCAGCTCCAGCCTACACA CCACCACCACCACCACCACCA C
o3W59HA	Used with o3HA46 to add the *ha* epitope sequence and *ftsW* homology to the disruption cassette	GGTTCCTCCGCCGGCGAGTAC GACATGCACCGAAATTCAGGC GTAGTCCGGGACGTC

Table 6 (continued)

Primer	Description	Sequence (5' → 3')
o3W63HIS	Used with o3HIS43 to add the *his*$_8$ epitope sequence and *ftsW* homology to the disruption cassette	GGTTCCTCCGCCGGCGAGTAC GACATGCACCGAAATTCAGTG GTGGTGGTGGTGGTGGTGGTG
o3W60	Used with o3HA46/o3W59HA or o3HIS43/o3W63HIS to add an additional site of *ftsW* homology to the disruption cassette	GCGACGGCGCGCCTCGGCGGC GCGTTCGTCCGGAGAGCGGAT TCCGGGGATCCGTCG
o3Q60HA	Used with o3HA46 to add the *ha* epitope sequence and *ftsQ* homology to the disruption cassette	GCTGCCCAACCAGGGTGCTGG CCTGCACGTATACCCGTCAGG CGTAGTCCGGGACGTCGT
o3Q60HIS	Used with o3HIS43 to add the *his*$_8$ epitope sequence and *ftsQ* homology to the disruption cassette	GCTGCCCAACCAGGGTGCTGG CCTGCACGTATACCCGTCAGT GGTGGTGGTGGTGGTGGTGGT G
o3Q60	Used with o3HA46/o3Q60HA or o3HIS43/o3Q60HIS to add an additional site of *ftsQ* homology to the disruption cassette	CGACGTGAGTGTTGCCACCGC CCCGGCGTCATCCGGGAGTAT TCCGGGGATCCGTCGACC
o3I60HA	Used with o3HA46 to add the *ha* epitope sequence and *ftsI* homology to the disruption cassette	TGGCTCGGTACGGCTCGGTGC GAGGGGGCGGTACGGCTCAG GCGTAGTCCGGGACGTCGT
o3I60HIS	Used with o3HIS43 to add the *his*$_8$ epitope sequence and *ftsI* homology to the disruption cassette	TGGCTCGGTACGGCTCGGTGC GAGGGGGCGGTACGGCTCAGT GGTGGTGGTGGTGGTGGTGGT G
o3I60	Used with o3HA46/o3I60HA or o3HIS43/o3I60HIS to add an additional site of *ftsI* homology to the disruption cassette	CGGGGCCGCCCCCGCGAGGCT CCCGGTCACCTTCAAACCCAT TCCGGGGATCCGTCGACC

PCR amplification of the disruption cassette with oligonucleotides containing gene homology and epitope tag sequences was preformed using an adaptation of a previously published protocol (Kolisnychenko *et al*., 2002). The conditions for the first reaction, the extension reaction, were as follows: 50 pmol universal primer, 50 pmol specific primer #1, 1X reaction buffer, 50 μM each dNTP, and 3.5 U DNA polymerase (Expand High Fidelity mixture, Roche Applied Science). PCR was performed using a

PTC-100 MJ Research Thermal Cycler (Global Medical Instrumentation, Inc.) with the following program: [94ºC - 40", 57ºC - 40", 72ºC - 15"] X 15 cycles, 4ºC - Hold. The conditions for the second reaction, the amplification reaction, were as follows: 2% extension reaction (unpurified, straight from prior reaction), 50 pmol gene specific primer #2, 50 ng template DNA (pIJ773 disruption cassette), 1X reaction buffer, 50 μM each dNTP, 5% dimethyl sulfoxide (DMSO), and 3.5 U DNA polymerase (same as previous reaction). PCR was performed using the following program: [94ºC - 40", 57ºC - 40", 72ºC - 80"] X 28 cycles, 72ºC - 10', 4ºC - hold.

These mutagenic PCR products were transformed into the *E. coli* strain BW25113/pIJ790/C69 by electroporation using a MicroPulser (Bio-Rad). The plasmid pIJ790 carries the λ RED genes *gam*, *bet*, and *exo* which prevent the *E. coli* exonuclease *recBCD* from degrading the cassettes and aid in homologous recombination between short sequences. These genes are under the control of the pBAD promoter and are induced by 10 mM L-arabinose when making the cells electrocompetent. The cosmid C69 carries the division and cell wall (*dcw*) gene cluster of the *S. coelicolor* chromosome, which contains all of the cell division genes used in this study. Cosmid derivatives resulting from these homologous recombination events are listed in Table 7.

Table 7

Derivatives of Cosmid C69 Created by Homologous Recombination between Modified pIJ773 Disruption Cassettes and Target Cell Division Genes

Cosmid	Description	Source or Reference
pJR155	*ha-frt-aac(3)IV-oriT-frt-ftsW*	McCormick, JR
pJR156	*his_8-frt-aac(3)IV-oriT-frt-ftsW*	McCormick, JR
pJR157	*ftsW-frt-aac(3)IV-oriT-frt-ha*	McCormick, JR

Table 7 (continued)

Cosmid	Description	Source or Reference
pJR158	*ftsW-frt-aac(3)IV-oriT-frt-his*$_8$	McCormick, JR
pJR159	*ha-frt -aac(3)IV-oriT-frt-ftsQ*	McCormick, JR
pJR160	*his*$_8$*-frt-aac(3)IV-oriT-frt-ftsQ*	McCormick, JR
pJR161	*ftsQ-frt-aac(3)IV-oriT-frt-ha*	McCormick, JR
pJR162	*ftsQ-frt-aac(3)IV-oriT-frt-his*$_8$	McCormick, JR
pJR163	*his*$_8$*-frt-aac(3)IV-oriT-frt-ftsZ*	McCormick, JR
pJR164	*ha-frt-aac(3)IV-oriT-frt-ftsI*	McCormick, JR
pJR165	*his*$_8$*-frt-aac(3)IV-oriT-frt-ftsI*	McCormick, JR
pJR166	*ftsI-frt-aac(3)IV-oriT-frt-ha*	McCormick, JR
pJR167	*ftsI-frt-aac(3)IV-oriT-frt-his*$_8$	McCormick, JR

FLP-Mediated DNA Recombination of Cosmid Derivatives

All cosmid derivatives listed in Table 7 containing the pIJ773 disruption cassette were transformed by electroporation using a MicroPulser (Bio-Rad) into the *E. coli* strain BT340, which contains the FLP recombinase-expressing plasmid pCP20. A single transformant was streaked for single colonies on an LB plate without antibiotic selection. These plates were grown for 16 hours at 42°C to induce the FLP recombinase gene. Single colonies were patched onto LB plates with and without apramycin. Cosmids that had undergone FLP-mediated DNA recombination were identified based on apramycin-sensitivity. These cosmids contain an epitope tag sequence and one 81 bp *frt* site scar sequence where the pIJ773 disruption cassette had been inserted. Some of these cosmid derivatives were previously created (McCormick JR, unpublished data). All cosmid derivatives created by this recombination event are listed in Table 8.

Table 8

Derivatives of Cosmid C69 Created by Removing the pIJ773 Disruption Cassette via FLP-Mediated DNA Recombination

Cosmid	Description	Source or Reference
pJR168	*his*$_8$*-frt-ftsW*	McCormick, JR
pJR169	*ftsW-frt-ha*	McCormick, JR
pJR170	*ftsW-frt-his*$_8$	McCormick, JR
pJR171	*ha-frt-ftsQ*	McCormick, JR
pJR172	*ftsQ-frt-ha*	McCormick, JR
pJR173	*ftsQ-frt-his*$_8$	McCormick, JR
pJR174	*his*$_8$*-frt-ftsZ*	McCormick, JR
pAK9	*his*$_8$*-frt-ftsQ*	This Study
pAK11	*ha-frt-ftsW*	This Study
pAK12	*ftsI-frt-his*$_8$	This Study
pAK13	*ha-frt-ftsI*	This Study
pAK14	*his*$_8$*-frt-ftsI*	This Study
pAK17	*ftsI-frt-ha*	This Study

PCR and Sequencing

PCR analysis and sequencing were performed on all cosmids after the FLP-mediated DNA recombination event to ensure that recombination occurred properly, leaving an 81 bp scar sequence and an epitope tag sequence with no deletions or base substitutions. Primer pairs used for amplification, using each individual cosmid as a template, are listed in Table 9. Verification of genes containing an epitope tag sequence via PCR was performed using a previously published protocol (Gust *et al*., 2002) (Figure S1A). Addition of *his*$_8$*-frt* or *ha-frt* to the cell division genes added 105 bp or 108 bp, respectively, to the expected size of the amplicon. The primers oI152 and oI167 did not amplify the correct region of the cosmids, resulting in multiple background bands of incorrect size (Figure S2). Because of this, the 5' end of *ftsI* in the cosmid derivatives pAK13 and pAK14 could not be sequenced and these constructs could not be completed.

New primers will have to be created to amplify the 5' end of *ftsI* correctly so that these constructs can be completed.

Table 9

Primers Used to Amplify the 5' and 3' Regions of Cell Division Genes

Primer Pair [a]	Description	Sequence (5' → 3')
oZ161/oZ132	Amplifies 293 bp at the 5' end of *ftsZ*	GTCGACTTAGTGTCCTGTTC/ TCGCTCATCAACAGCGCCTG
oW164/oW170	Amplifies 334 bp at the 5' end of *ftsW*	GCACGGTGATCAGCGCACTG/ CCGGTGACACGGTGCTGCTG
oW119/oW156	Amplifies 275 bp at the 3' end of *ftsW*	GTCTCCAGGCCGCGTTCCGTG/ TCGGTCTGCTGATCGCCTTC
oI152/oI167	Amplifies 319 bp at the 5' end of *ftsI*	GAGGCGCTGGCCTCGACGTC/ GGCCGATCATGCGCAGCCTG
oI144/oI139	Amplifies 283 bp at the 3' end of *ftsI*	TCACCGTCTACTGCGCCATC/ GGTGAGCGTACCGGCAGTAC
oQ160/oQ161	Amplifies 321 bp at the 5' end of *ftsQ*	CGGAGTGGCTCCAGCAGAAC/ GTTCCAGACGTGTCCAGTTC
oQ174/oQ165	Amplifies 339 bp at the 3' end of *ftsQ*	ACACGCGGGTCGTCAAGGTC/ CCCGAACCCTAACGCTGAAG

a) The primer pair oI152/oI167 did not amplify the 5' end of *ftsI* properly.

The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for all sequencing reactions. The reactions were analyzed using an ABI Prism 310 Genetic Analyzer and the sequences produced were analyzed using the DNA and protein sequence analyzer program MacVector (MacVector, Inc.). The portions of the cell division genes sequenced were compared to wildtype sequences submitted to the *S. coelicolor* A3(2) Genome Database (Figure S1B).

Making Cosmid Derivatives Mobilizable

All cosmid derivatives listed in Table 8 were made mobilizable using an insertion-deletion cassette, obtained from the plasmid pIJ799, which replaces the ampicillin-resistance gene (*bla*), a marker that cannot be used for selection in *Streptomyces*. This disruption cassette contains two sequences homologous to *bla* that flank *aac(3)IV* and *oriT*. This 1321 bp cassette was removed from pIJ799 by digesting the plasmid with *Eco*RI and *Hin*dIII and gel purifying the fragment. This cassette was transformed into the *E. coli* strain BW25113/pIJ790, which carried one of the cosmids from Table 8. The *bla* gene found in the backbone of these cosmids was deleted and an apramycin resistance gene that is functional in both *E. coli* and *S. coelicolor* was added in its place. This cassette also contains an *oriT* site. Mobilizable cosmids created are listed in Table 10. Plasmids pAK13 and pAK14 were not made mobilizable because the 5' end of *ftsI* could not be verified via PCR or sequencing analyses after FLP-mediated DNA recombination. All other mobilizable cosmid derivatives were verified via restriction enzyme digestion analysis using the restriction enzymes *Sac*I and *Xho*I (Figure S3). The gel electrophoresis pattern of restriction enzyme-digested fragments for each cosmid was compared to the pattern of pAK2, which served as the positive control.

Table 10

Mobilizable Cosmids Created by Replacing the Ampicillin-Resistance Gene with the pIJ799 Disruption Cassette

Cosmid [a]	Derived From	Gene Fusion Within Cosmid	Description	Source or Reference
pAK1	pJR174	*his*$_8$*-frt-ftsZ*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK2	C69	N/A	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK3	pJR168	*his*$_8$*-frt-ftsW*	Δ*bla*::*aac(3)IV-oriT*	This Study

Table 10 (continued)

Cosmid [a]	Derived From	Gene Fusion Within Cosmid	Description	Source or Reference
pAK4	pJR169	*ftsW-frt-ha*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK5	pJR170	*ftsW-frt-his*$_8$	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK6	pJR171	*ha-frt-ftsQ*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK7	pJR172	*ftsQ-frt-ha*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK8	pJR173	*ftsQ-frt-his*$_8$	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK10	pAK9	*his*$_8$*-frt-ftsQ*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK15	pAK11	*ha-frt-ftsW*	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK16	pAK12	*ftsI-frt-his*$_8$	Δ*bla*::*aac(3)IV-oriT*	This Study
pAK19	pAK17	*ftsI-frt-ha*	Δ*bla*::*aac(3)IV-oriT*	This Study

a) The cosmid pAK2 was created only to be used as a positive control during restriction enzyme digest analyses.

Interspecies Conjugation between *E. coli* and *S. coelicolor*

Interspecies conjugation and homologous recombination were used to incorporate each cosmid into the chromosome of wildtype *S. coelicolor*. Each mobilizable cosmid was first transformed into the *E. coli* strain ET12567/pUZ8002 by electroporation using a MicroPulser (Bio-Rad). A single colony was incubated overnight at 37°C with aeration in 3 ml of LB containing kanamycin, apramycin, and chloramphenicol. This culture was diluted 1:100 into 20 ml fresh LB containing the same antibiotics and incubated with aeration at 37°C for 4-5 hours. One-fourth of the culture was removed and pelleted by centrifugation. The cells were washed twice with LB containing no antibiotics and resuspended in 500 μl of LB. While washing the *E. coli* cells, 100 μl of spores of the wildtype strain M145 were pelleted and washed once with 5.73% N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.2. These spores were added to 500 μl 2X YT and heat shocked at 50°C for 10 minutes. Once the spores cooled to room temperature, they were added to the 500 μl of *E. coli* cells and mixed by

vortexing. The cells and spores were pelleted by briefly micro-centrifuging and the supernatant was removed by aspiration. The pellet was resuspended in 250 µl of LB and plated onto SFM agar without antibiotic selection. These plates were incubated overnight at 30°C. The next day, the plates were overlayed with 1 ml of water containing 0.5 mg nalidixic acid, to select against the *E. coli* donor strain, and 1.25 mg apramycin, to select transconjugants. Three to five days later, colonies were restreaked onto SFM agar containing nalidixic acid and apramycin to ensure that the cosmid incorporated into the *S. coelicolor* chromosome. Strains were further verified by PCR and/or sequencing analyses (Figure S4). New merodiploid *S. coelicolor* strains created by homologous recombination after conjugation are listed in Table 11.

Table 11

***S. coelicolor* Strains Created by Homologous Recombination after Conjugation**

Bacterial Strain[a]	Description[b]	Source or Reference
AK1	*dcw*::pAK1 (heterozygous: *ftsZ*$^+$/*his*$_8$-*frt*-*ftsZ*)	This Study
AK2	*dcw*::pAK3 (homozygous: *his*$_8$-*frt*-*ftsW*/*his*$_8$-*frt*-*ftsW*)	This Study
AK3	*dcw*::pAK5 (heterozygous: *ftsW*$^+$/*ftsW*-*frt*-*his*$_8$)	This Study
AK5	*dcw*::pAK15 (heterozygous: *ftsW*$^+$/*ha*-*frt*-*ftsW*)	This Study
AK6	*dcw*::pAK15 (homozygous: *ha*-*frt*-*ftsW*/*ha*-*frt*-*ftsW*)	This Study
AK7	*dcw*::pAK4 (heterozygous: *ftsW*$^+$/*ftsW*-*frt*-*ha*)	This Study
AK8	*dcw*::pAK6 (heterozygous: *ftsQ*$^+$/*ha*-*ftsQ*)	This Study
AK9	*dcw*::pAK7 (homozygous: *ftsQ*-*frt*-*ha*/*ftsQ*-*frt*-*ha*)	This Study
AK10	*dcw*::pAK8 (homozygous: *ftsQ*-*frt*-*his*$_8$/*ftsQ*-*frt*-*his*$_8$)	This Study

Table 11 (continued)

Bacterial Strain[a]	Description[b]	Source or Reference
AK11	*dcw*::pAK8 (heterozygous *ftsQ*$^+$/*ftsQ-frt-his*$_8$)	This Study
AK12	*dcw*::pAK6 (homozygous: *ha-frt-ftsQ*/*ha-frt-ftsQ*)	This Study
AK16	*dcw*::pAK10 (homozygous: *his*$_8$*-frt-ftsQ*/*his*$_8$*-frt-ftsQ*)	This Study
AK17	*dcw*::pAK10 (heterozygous *ftsQ*$^+$/*his*$_8$*-frt-ftsQ*)	This Study
AK18	*dcw*::pAK1 (homozygous: *his*$_8$*-frt-ftsZ*/*his*$_8$*-frt-ftsZ*)	This Study
AK19	*dcw*::pAK19 (homozygous: *ftsI-frt-ha*/*ftsI-frt-ha*)	This Study
AK21	*dcw*::pAK19 (heterozygous: *ftsI*$^+$/*ftsI-frt-ha*)	This Study
AK22	*dcw*::pAK16 (heterozygous: *ftsI*$^+$/*ftsI-frt-his*$_8$)	This Study
AK23	*dcw*::pAK5 (homozygous: *ftsW-frt-his*$_8$/*ftsW-frt-his*$_8$)	This Study

a) All strains are derived from M145. b) Status of the markers was analyzed by PCR; Gene conversion events occur within approximately 10% of all transconjugants; *dcw* - division and cell wall gene cluster of the chromosome

Intramolecular Homologous Recombination between Duplicate *dcw* Gene Clusters

To remove the duplicate *dcw* gene cluster without the epitope-tagged gene, strains were streaked on SFM agar without antibiotic selection. Candidates that had undergone intramolecular homologous recombination were chosen based on sensitivity to apramycin and kanamycin, the antibiotic resistant markers in the cosmid backbone. Strains were further verified by PCR, sequencing, and/or Southern blot hybridization analyses. All completed strains are listed in Table 12.

Table 12

Final *S. coelicolor* Strains Created via Intramolecular Homologous Recombination between Duplicate *dcw* Gene Clusters

Bacterial Strain	Derived From	Description[a]	Source or Reference
AK4	AK2	*his*$_8$*-frt-ftsW*	This Study
AK13	AK12	*ha-frt-ftsQ*	This Study
AK14	AK9	*ftsQ-frt-ha*	This Study
AK15	AK10	*ftsQ-frt-his*$_8$	This Study
AK20	AK6	*ha-frt-ftsW*	This Study

a) The epitope-tagged gene listed is the only copy of that gene found in the chromosome.

Southern Blot Hybridization Analysis

Southern blot hybridization analysis was performed using DNA isolated from intramolecular homologous recombination candidates to ensure that rearrangements or deletions had not occurred within the division and cell well cluster during the creation of the new *S. coelicolor* strains. These strains were compared to the wildtype strain M145. Chromosomal DNA from all strains was digested separately with three different restriction enzymes (*Sac*I, *Xho*I, or *Pst*I), which do not have recognition sites within the epitope-tag sequence. The cosmid C69 was digested with *Xho*I and all fragments created, ranging in size from 54 bp to 15 kb, were used as probes for the Southern blot. The probes were nonisotopically labeled and hybrid bands were detected immunologically (DIG DNA Labeling and Detection Kit, Boehringer Mannheim). Hybridization was performed at 65°C in buffer that contained 5% SDS (Virca *et al*., 1990). A stringent post-hybridization wash of 0.1% SSC was performed at 65°C.

Phase-Contract Microscopy

S. coelicolor strains were grown on MM, R2YE, or SFM agar for phase-contrast microscopy. Coverslip lifts were prepared by inserting sterile coverslips into the solid agar media at a 45° angle. The coverslips were inoculated along the adjacent agar surface with the desired strain. After five days of growth, the coverslips were removed and mounted in 50% glycerol for observation. Microscopy was performed using a Nikon Microphot-SA microscope with a 100X oil immersion lens. Images were captured using a DC 290 Zoom Digital Camera (Kodak).

Preparation of *S. coelicolor* Whole Cell Extracts

Strains were grown in 30 ml of YEME for 2-3 days at 30°C with aeration at 250 rpm. Mycelia were harvested by centrifugation and washed 3 times in 10.3% sucrose. The final pellet was resuspended in 1 ml of sonication buffer (300 mM NaCl, 50 mM Na-phosphate, pH 8.0). Before sonication, 10 µl/ml of protease inhibitor cocktail (Sigma P8849) was added to each sample. The resuspended pellets were subjected to sonication at 10 Watts by a Sonic Dismembrator (Fisher Scientific) while incubated on ice. Three minutes of sonication were applied to all samples, alternating 10 seconds burst and 20 seconds cooling periods. The samples were centrifuged at maximum speed for 20 minutes at 4°C (Eppendorf MicroCentrifuge 5402). The supernatant was collected and stored at -80°C. All samples were analyzed by SDS-PAGE and western blot analyses.

Fractionation of *S. coelicolor* Lysate via High Speed Centrifugation

Strains were grown in 500 ml of YEME at 30°C for 48-72 hours. Mycelia were pelleted by centrifugation at 5000 rpm for 10 minutes in the Sorval SS34 rotor at 4°C and washed 3 times with 10.3% sucrose. All samples were kept at 4°C from this point forward. The pellets were resuspended in 3-5 ml ice-cold lysis buffer (25% sucrose, 10 mM Tris-HCl, pH 7.5). Protease inhibitor cocktail (Sigma P8849) was added to each sample at a final concentration of 10 µl/ml. Cells were lysed by twice passing the resuspended pellet through the French Press (Thermo Spectronic) at 15,000 psi. The French Pressure cell was incubated on ice for 1 hour before use. After lysing the cells, DNase I (Roche) and RNase A (Boehringer Mannheiem) were added to a final concentration of 10 µg/ml each. To remove unlysed cells and other cellular debris, samples were centrifuged at 3,500 rpm for 10 minutes at 4°C in a Sorval SS34 rotor. The supernatant was transferred to an Oak Ridge centrifuge tube (Nalgene) and centrifuged in a Beckman XL-70 ultra-centrifuge using the TY 70.1 Ti rotor at 45,000 rpm (200,000 xg) for 60 minutes at 4°C. The supernatant, which contains soluble proteins, was collected, flash frozen with liquid nitrogen, and stored at -80°C. The pellet, which contains mainly membrane-bound proteins, was resuspended in 500 µl solubilizing buffer (25% sucrose, 1% Nonidet-P40, 10 mM Tris-HCl, pH 7.5) to remove proteins from the membrane. Protease inhibitor cocktail (Sigma P8849) was added at a concentration of 10 µl/ml. Once the pellet was resuspended, the solubilized membrane fractions were centrifuged at 12,000 rpm for 10 minutes at 4°C (Eppendorf MicroCentrifuge 5402). The supernatant containing solubilized membrane proteins was collected and aliquoted into 100 µl

samples. All samples were flash frozen in liquid nitrogen and stored at -80°C. These fractions were used in nickel-nitrilotriacetic acid (Ni-NTA) pull down assays.

Ni-NTA Pull Down Assay

This method was adapted from a protocol provided by Dr. Jeffrey Brodsky (University of Pittsburgh; Pittsburgh, PA). All samples and buffers were kept at 4°C. A solubilized membrane fraction was slowly thawed on ice and brought up to 800 µl with ice-cold solubilizing buffer. This sample was added to 200 µl of 50% (w/v) slurry Ni-NTA agarose (Qiagen) that had previously been equilibrated in ice-cold solubilizing buffer. The samples were incubated for 1 hour while mixing at 4°C on an Adams Nutator (BD). The agarose was pelleted by gently micro-centrifuging at minimum speed for 20 seconds at 4°C (Eppendorf MicroCentrifuge 5402). The supernatant was collected and saved. This supernatant fraction was referred to as "No Bind". The agarose beads were washed once with 1 ml solubilizing buffer, three times with 1 ml high salt buffer (0.5 M NaCl, 25% sucrose, 1% Nonidet-P40, 10 mM Tris-HCl, pH 7.5), and three times with 1 ml imidazole buffer (500 mM imidazole, 25% sucrose, 1% Nonidet-P40, 10 mM Tris-HCl, pH 7.5). The supernatants of all washes were collected after gentle centrifugation and were named "Buffer Wash", "Salt Wash 1", "Salt Wash 2", "Salt Wash 3", "Imidazole Wash 1", "Imidazole Wash 2", and "Imidazole Wash 3", respectively. Approximately 100 µl of 2X Laemmli sample buffer (10% SDS, 50% glycerol, 0.5% bromophenol blue, 0.5 M Tris-HCl, pH 6.8, 0.5% β-mercaptoethanol) was added to the beads after the last wash and incubated at 90°C for five minutes or 50°C for 15 minutes to remove any proteins still bound to the Ni-NTA agarose. This fraction was referred to

as “Bead Boil”. All wash fractions were concentrated by tricloroacetic acid (TCA) precipitation.

TCA Precipitation

Ice-cold 100% TCA was added to each wash fraction to a final concentration of 20% and mixed by vortexing. Samples were incubated on ice for 30 minutes and centrifuged for 10 minutes at maximum speed at 4°C (Eppendorf MicroCentrifuge 5402). The supernatant was removed by aspiration and the pellet was washed by vortexing in ice-cold acetone. Samples were immediately centrifuged again for 10 minutes at maximum speed at 4°C. The acetone was removed by aspiration and the pellet was resuspended in 20-50 µl of 1X Laemmli sample buffer. If the bromophenol blue in the sample buffer turned yellow, it was neutralized by adding 1-10 µl of 1 M Tris-Cl, pH 7.5. All samples were incubated at 90°C for five minutes or 50°C for 15 minutes and analyzed by SDS-PAGE or western blot analyses.

Protein Concentration Determination

The Bradford assay method (Bio-Rad Protein Assay) was utilized to determine the concentration of proteins in each sample. The reading obtained was compared to a standard curve that allowed for a relative measurement of protein concentration. The standard curve was produced by using varying concentrations of bovine serum albumin (BSA).

SDS-PAGE

SDS-PAGE was performed using the Mini-PROTEAN III electrophoresis system (Bio-Rad). Samples were fractionated on 8, 10, or 12% polyacrylamide gels along with either BenchMark protein ladder (Invitrogen), MagicMark western protein standard (Invitrogen), or MagicMark XP western protein standard (Invitrogen). Gels were electrophoresed between 100 and 150 volts in running buffer (3% Tris-HCl, 14.4% glycine, 1% SDS) until the bromophenol blue dye reached the bottom of the gel. Gels were either electrotransferred to PVDF membranes, stained with Commassie stain (0.125% Commassie blue R-250, 50% methanol, 10% acetic acid), or stained with nickel (Nickel Staining Kit, Fisher Scientific).

Western Blot Analysis

The Mini Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad) was used for western blot experiments. Proteins fractionated by SDS-PAGE were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). The transfer was carried out in transfer buffer (192 mM glycine, 20% v/v methanol, 25 mM Tris-HCl, pH 8.3). The protein transfer was carried out at 100 V for 1 hour at 4°C with buffer recirculation. All of the remaining steps were carried out at room temperature.

After transfer, the membranes were blocked with a BSA blocking solution (5% BSA, 150 mM NaCl, 0.05% Tween 20, 20 mM Tris-HCl, pH 7.5) or a milk blocking solution (5% powdered milk, 150 mM NaCl, 0.05% Tween 20, 20 mM Tris-HCl, pH 7.5). The membranes were incubated in appropriate dilutions of primary antibody in TBST (150 mM NaCl, 0.05% Tween 20, 20 mM Tris-HCl, pH 7.5) for 1 hour, or

overnight at 4°C, with constant shaking. The primary antibodies used and the appropriate dilutions are as follows: rabbit polyclonal α-FtsZ - 1:50,000 (Schwedock *et al*., 1997); rabbit polyclonal α-FtsI - 1:100,000 (Yarnall, 2001); rabbit polyclonal α-FtsQ - 1:100,000 (Stadelmaier B, Kuennen R, and McCormick JR, unpublished data), mouse monoclonal α-His_5 - 1:1,000 (Qiagen), rabbit polyclonal α-His - 1:200 (Santa Cruz Biotechnology). The membranes were washed twice with TBST for 7 minutes and once with TBST for 5 minutes to remove unbound primary antibody.

For colorimetric detection, the secondary antibody, alkaline phosphatase-conjugated α-rabbit IgG (Promega), was incubated with the membrane at a 1:5,000 dilution in TBST for 1 hour. The membrane was washed with TBST as before to remove any unbound secondary antibody. An additional 30 second wash with TBS was carried out. The antibodies were visualized by adding the color development substrates, Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Promega), in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5). The reactions were terminated by washing the membranes in water.

For chemiluminescence detection, the secondary antibody, horseradish peroxidase-conjugated α-mouse IgG (Pierce), was incubated with the membrane at a 1:10,000 dilution in TBST for 1 hour. The membrane was washed with TBST as before to remove any unbound secondary antibody. An additional 30 second wash with TBS was carried out. The antibodies were visualized by mixing the chemiluminescence development substrates, Peroxide Buffer and Luminol/Enhancer Solution (Pierce), in a 1:1 ratio and incubating this mixture on the membrane for 5 minutes. The membrane was

immediately scanned using a Typhoon 8600 Variable Mode Imager following manufacturer's instructions (Amersham Biosciences).

Partial Purification of Polyclonal Antibodies

To partially purify the α-FtsZ, α-FtsI, and α-FtsQ antibodies, whole cell extract from the *ftsZ*-, *ftsI*-, and *ftsQ*-null *S. coelicolor* strains was fractionated by SDS-PAGE and electrotransferred onto a PVDF membrane. The membranes were blocked and washed as described before. Next, the appropriate dilution of each antibody was applied to the membrane that contained the whole cell extract lacking the protein of interest. The membrane was incubated overnight at room temperature or 4°C with constant shaking to remove background reactive antibodies. The next day, the primary antibody solution, now lacking nonspecific antibodies, was removed and stored at 4°C until needed. The membrane was analyzed via the colorimetric detection described above.

Overexpression and Purification of the C-Terminal Domain of FtsI

A previously made plasmid, pJY1, was used to overexpress *ftsI* (Yarnall, 2001). This gene was truncated at the 5' end, removing the region coding for the single membrane-spanning region of the protein. A his_6 sequence and the T7 promoter were added in its place. This plasmid was transformed into the *E. coli* strain BL21(DE3)/pLysS, which allows for overexpression of His_6-'FtsI upon induction by isopropyl β-D-1-thiogalactopyranoside (IPTG). This *E. coli* strain was provided by Dr. Mohammed Qutyan (Duquesne University; Pittsburgh, PA).

To overexpress this protein, 500 ml of LB containing chloramphenicol and carbenicillin was inoculated with 0.5 ml of an overnight culture containing BL21(DE3)/pLysS/pJY1. The cells were grown at 37°C with constant aeration until the culture reached an OD_{600} of 0.5. IPTG was added to a final concentration of 1 mM. Cells were grown for an additional 4-5 hours at 37°C with constant aeration. The cultures were pelleted by centrifugation at 5000 rpm for 10 minutes in the Sorval SS34 rotor at 4°C. The supernatant was decanted and the pellet was weighed and stored at -80°C until the proteins could be purified.

Purification of proteins was carried out under denaturing conditions using a Ni-NTA affinity column (Qiagen) following manufacturer's instructions. Fractions were analyzed via SDS-PAGE analysis and pooled according to elution buffer. The proteins were concentrated using a Centriprep-10 Centrifugal Filter Unit (Millipore) and a Centricon-10 Centrifugal Filter Unit (Millipore) following manufacturer's instructions. Purified, concentrated protein samples were stored at -80°C until needed.

RESULTS

The purpose of this thesis was to begin to biochemically analyze putative cell division protein interactions in *S. coelicolor* to complement and possibly expand upon previous genetic analyses performed in this laboratory. This study utilized and built upon constructs that were previously created in the laboratory (McCormick JR, unpublished data). The proteins FtsZ, FtsQ, FtsW, and FtsI were analyzed to determine if cell division protein subcomplexes are found in *S. coelicolor* and to possibly determine if additional interacting proteins exist. Before a biochemical analysis could be conducted, several tools were needed. First, *S. coelicolor* strains that express epitope-tagged cell division proteins were created. Two different epitope tags were used: an eight-histidine (His_8) epitope tag to isolate proteins and a hemagglutinin (HA) epitope tag to visualize proteins via western blot analysis. Additional polyclonal antibodies specific to FtsZ, FtsQ, and FtsI have previously been created and were utilized in this study (Schwedock *et al*., 1997; Stadelmaier B, Kuennen R, and McCormick JR, unpublished data; Yarnall, 2001).

Creation of *S. coelicolor* Strains that Express Epitope-Tagged FtsZ

The first *S. coelicolor* protein to be epitope-tagged and analyzed was FtsZ. Only the N-terminus of the protein was tagged, because it has been previously discovered that the C-terminus is required for proper function (Morris, 2002). FtsZ was selected because it is abundant and cytoplasmic, which allows for easy analysis. This protein was also chosen because highly specific polyclonal antibodies were available to visualize it via

western blot analysis. A His_8-FtsZ fusion protein was created and used to test the binding ability and the stability of the epitope tag via western blot analysis.

Prior to my involvement in this project, the incorporation of the *his*$_8$ epitope tag sequence into *ftsZ* was carried out by manipulation of the cosmid C69, which contains the division and cell wall (*dcw*) gene cluster of *S. coelicolor* (McCormick JR, unpublished data). A disruption cassette isolated from pIJ773 was utilized for this purpose. Before this cassette could be used, the *his*$_8$ epitope tag sequence and homology to the 5' end of *ftsZ* was added to the cassette using two different PCR reactions. In the extension reaction, two primers, the universal primer oHIS44, which can be used to His_8-Tag the 5' end of any gene, and the *ftsZ* specific primer oZ64HIS, were used, without template DNA, to create a short double-stranded DNA (dsDNA) segment (Figure 3A). The resulting dsDNA molecule contained homology to the start codon of *ftsZ*, as well as 37 bp of upstream sequence, homology to the pIJ773 disruption cassette, and the *his*$_8$ epitope tag sequence. The short dsDNA segment and the pIJ773 cassette overlap and prime each other in the early part of the second PCR reaction, the amplification reaction. The *ftsZ* specific primers oZ64HIS and oZ59, which contained homology to the second codon of *ftsZ* and 56 bp of downstream sequence, were utilized to amplify the joined *his*$_8$-pIJ773 cassette (Figure 3B). Both PCR reactions resulted in a modified disruption cassette that contained the *his*$_8$ epitope tag sequence and homology to the 5' end of *ftsZ* (Figure 3C).

This disruption cassette, containing a gene specifying apramycin-resistance, was transformed into the *E. coli* strain BW25113/pIJ790/C69. The modified pIJ773 cassette was incorporated into C69 via homologous recombination (Figure 4A). The λ RED recombination plasmid pIJ790 was utilized to help this process. This plasmid expresses

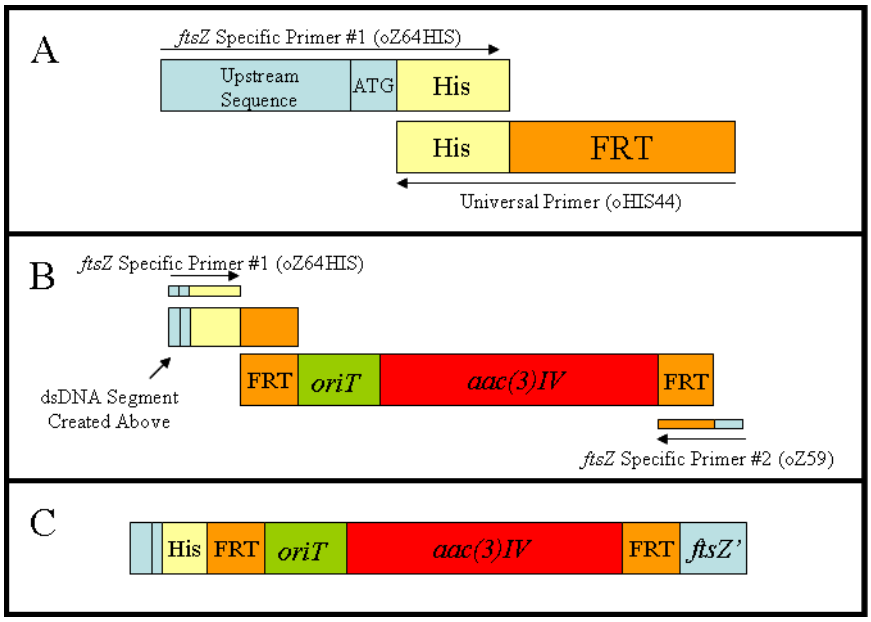

Figure 3

Creation of a disruption cassette to incorporate the *his*$_8$ epitope tag sequence into *ftsZ*. A) Primers oHIS44 and oZ64HIS were used to create a short dsDNA segment during an extension PCR reaction. B) The newly created dsDNA molecule and the primers oZ64HIS and oZ59 were utilized in an amplification PCR reaction to add the *his*$_8$ epitope-tag sequence and homology to the 5' end of *ftsZ* to the pIJ773 disruption cassette. C) The newly created disruption cassette after the two PCR reactions.

the functions *exo*, *bet*, and *gam*, which prevent the degradation of the linear disruption cassette and also aid in homologous recombination with short sequences. The resulting cosmid, pJR163, was isolated and transformed into the *E. coli* strain BT340, a strain that carries the FLP recombinase-expressing plasmid pCP20. FLP-recombinase was induced to cause a site-specific recombination event, this time between the two *frt* sites within pJR163, removing the *aac(3)IV* gene (Figure 4B). The resulting cosmid, pJR174, possesses the *his*$_8$ epitope tag sequence and one 81 bp *frt* site scar sequence where the disruption cassette was inserted. To ensure that the FLP-mediated DNA recombination event happened correctly, without base pair substitution or deletion, the 5' end of *ftsZ* was analyzed via PCR and sequencing analysis (For an example see Figure S1).

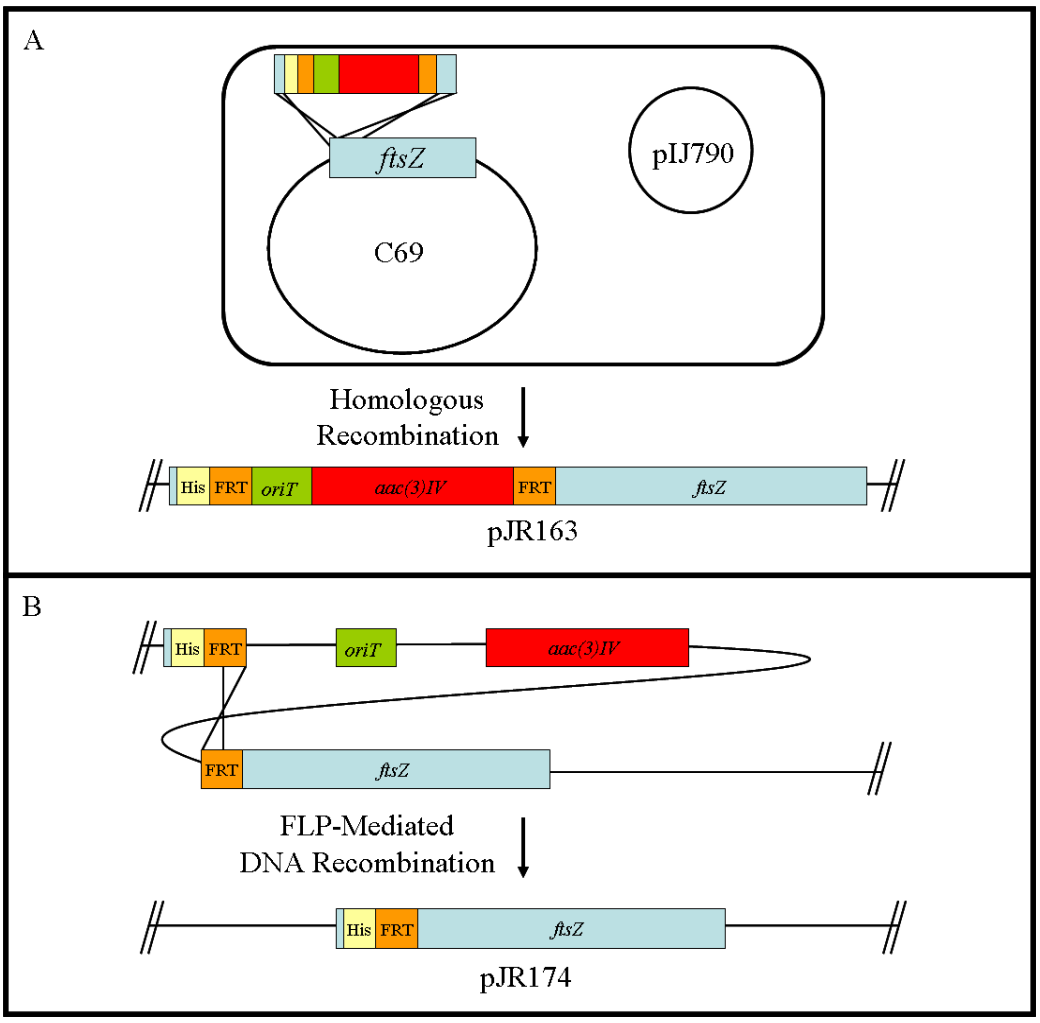

Figure 4

Incorporation of the *his*$_8$ epitope tag sequence into *ftsZ*. A) The modified pIJ773 disruption cassette (Figure 3C) was incorporated into *ftsZ* via homologous recombination with the aid of the λ RED recombination plasmid pIJ790. B) Most of the disruption cassette was removed from *ftsZ* via FLP-mediated DNA recombination, leaving the *his*$_8$ epitope tag sequence and an 81 bp *frt* site scar sequence between the first and second codons of *ftsZ*.

Next, the cosmid pJR174 was made mobilizable to allow conjugation between *E. coli* and *S. coelicolor* and to replace the ampicillin-resistance marker that is unusable for selection in *S. coelicolor*. An insertion-deletion cassette isolated from pIJ799 was used for this purpose by transforming it into the *E. coli* strain BW25113/pIJ790/pJR174

(Figure 5). This cassette contained *aac(3)IV* and *oriT* flanked by two sites of *bla* homology. A homologous recombination event between the cassette and *bla* was aided again by the λ RED recombination plasmid pIJ790. The resulting cosmid, pAK1, was isolated and transformed into the *E. coli* strain ET12567/pUZ8002. This strain allows intraspecies conjugation between *E. coli* and *S. coelicolor*. The entire cosmid pAK1 was transferred and then incorporated in the chromosome of the wildtype *S. coelicolor* strain M145 via homologous recombination (Figure 6A). Kanamycin- and apramycin-resistant transconjugants were selected. This selection was carried out to ensure that the cosmid pAK1 incorporated into the *S. coelicolor* chromosome in its entirety via a single

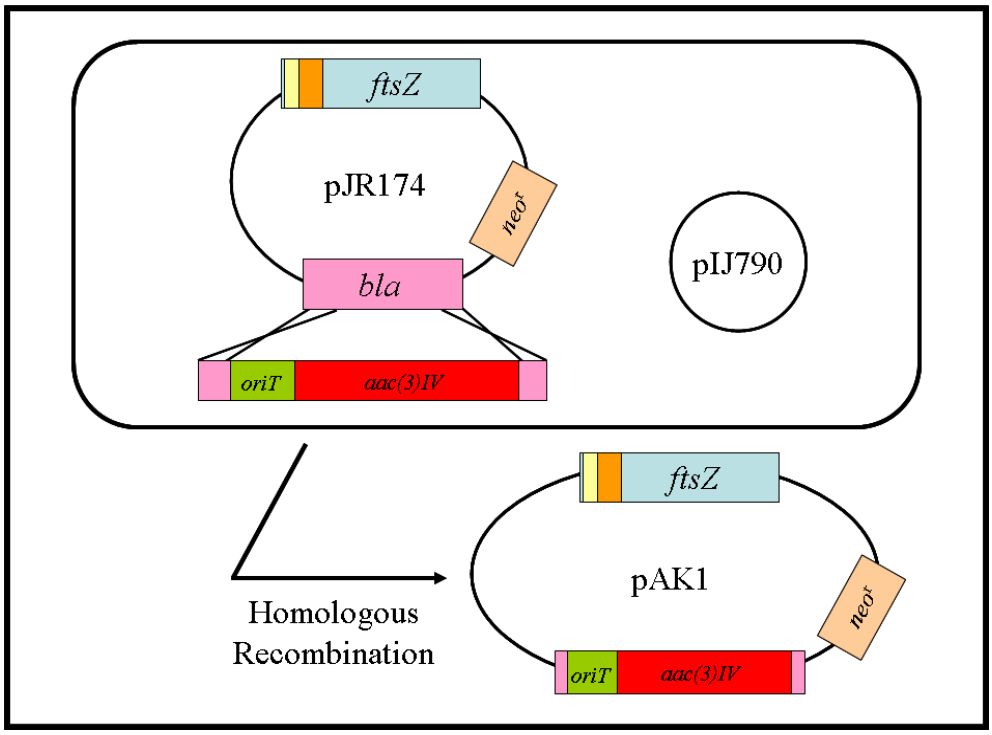

Figure 5

Strategy for making cosmid derivatives mobilizable. In this example, cosmid pJR174 was made mobilizable by replacing *bla* with *aac(3)IV* and *oriT* by *in vivo* recombination, resulting in the creation of pAK1. This strategy was used to make all cosmid derivatives mobilizable. The *neo*^{r} gene confers resistance to kanamycin.

homologous recombination event. All transconjugants contain a duplication of the *dcw* gene cluster and therefore two copies of *ftsZ*. Two strains, AK1 and AK18, were isolated from this conjugation experiment and were further analyzed (Figure 6B).

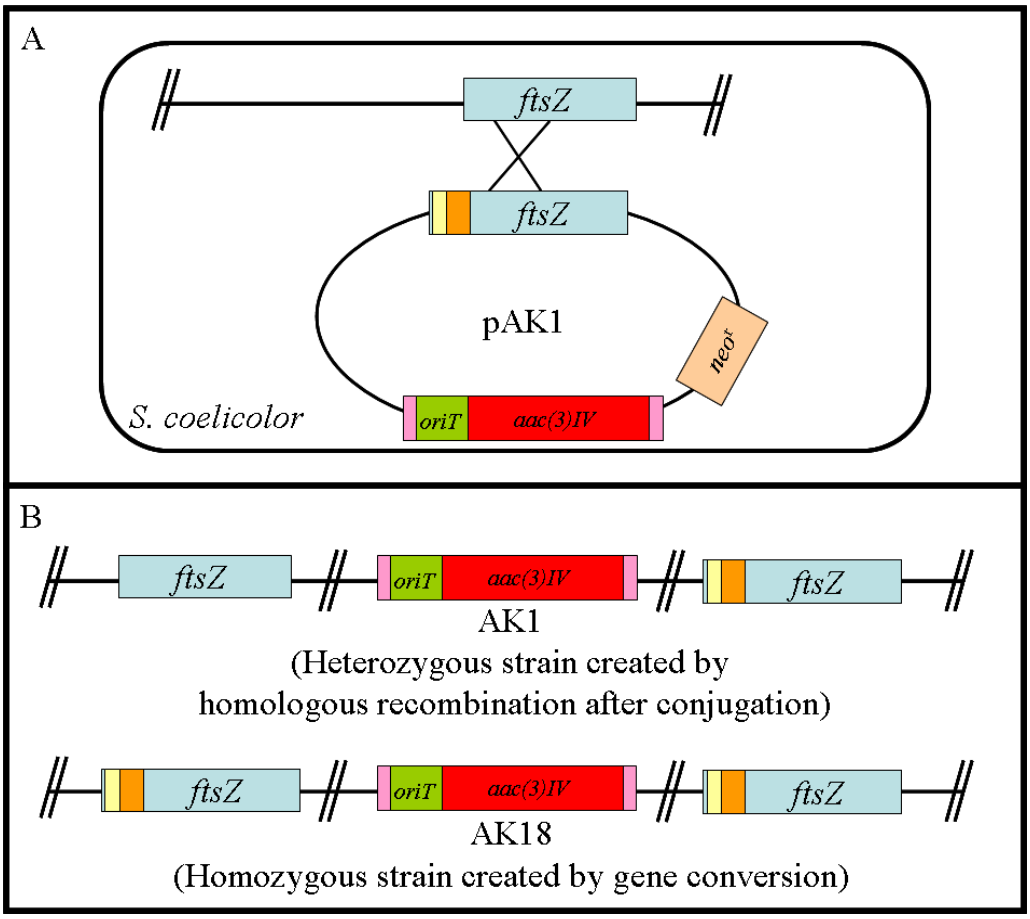

Figure 6

Incorporation of pAK1 into the *S. coelicolor* chromosome via homologous recombination after conjugation. A) The cosmid pAK1 was incorporated into the chromosome of the wildtype *S. coelicolor* strain M145 after conjugation by a single homologous recombination event. The homologous recombination event pictured was drawn as occurring in *ftsZ* for simplicity sake. This event can actually happen at any region of the pAK1 insert that is homologous to the *S. coelicolor* chromosome. B) Strains AK1 and AK18 were two transconjugants isolated from this conjugation event. PCR analysis indicated that AK18 contains two copies of his_8-*ftsZ* because of a gene conversion event between the two copies of *ftsZ* (see text). The neo^r gene confers resistance to kanamycin.

Analysis of *S. coelicolor* that Express Epitope-Tagged FtsZ

Each transconjugant was analyzed via PCR to verify that it carried two copies of *ftsZ*, the wildtype copy and the copy that contained the *his*$_8$ epitope tag sequence (For an example see Figure S4). While most strains were found to be heterozygous (*ftsZ*$^+$/*his*$_8$-*ftsZ*), other strains had undergone gene conversion and were found to be homozygous (*ftsZ*$^+$/*ftsZ*$^+$ or *his*$_8$-*ftsZ*/*his*$_8$-*ftsZ*). Two strains, AK1 and AK18, were selected for further analysis based on these PCR results (Figure 7A). AK1 was found to be heterozygous and AK18 was found to be homozygous for *his*$_8$-*ftsZ*. To verify that AK1 and AK18 were indeed heterozygous and homozygous, respectively, whole cell extract was prepared from these strains, as well as the wildtype strain M145 and the *ftsZ*-null strain HU133, and analyzed by western blot analysis (Figure 7C). This western blot analysis was also carried out to determine if the epitope tag and scar peptide on FtsZ was stable. To remove nonspecific antibodies from the serum, the α-FtsZ antibodies were first incubated with a membrane that contained whole cell extract from the *ftsZ*-null strain HU133 (Figure 7B). FtsZ was visible in the wildtype sample, migrating at about 49 kDa and is absent in lane that corresponds to the *ftsZ*-null mutant HU133 (Figure 7C). Two different bands of close molecular weight, 49 kDa and 52 kDa, could clearly be seen in the lane that corresponds to AK1, verifying the earlier PCR result that this strain is heterozygous for *ftsZ*. While the two bands in this lane appear to have different intensities, in other analyses both bands had similar intensities (See Figures S6B and S8B). The fact that AK18 was homozygous for *his*$_8$-*ftsZ* was found to be true, as only the 52 kDa band appeared in the lane corresponding to this strain. This proved that the His$_8$ epitope tag and the 21 amino acid scar peptide are stable *in vivo*.

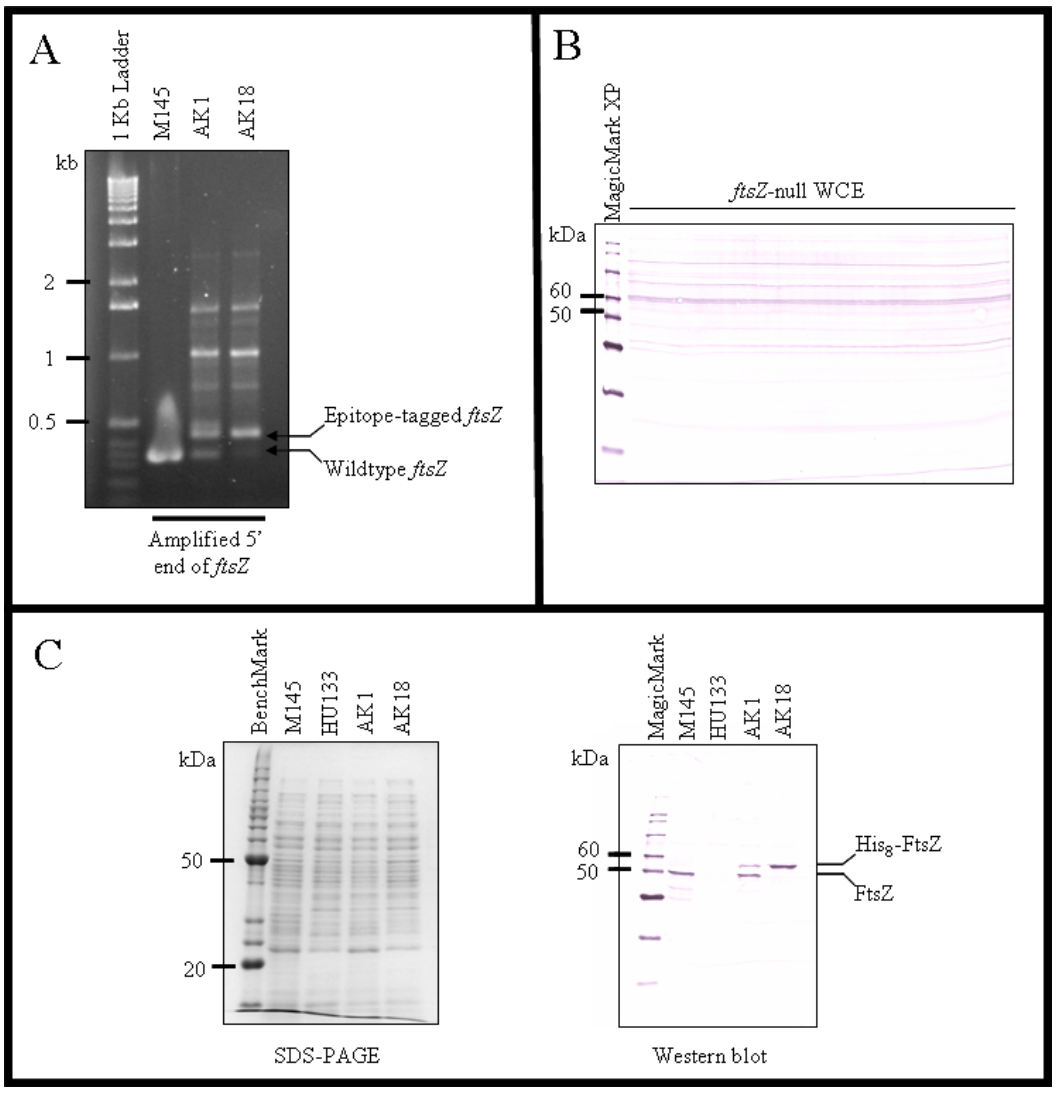

Figure 7

PCR and western blot analyses of *S. coelicolor* strains AK1 and AK18. A) Primers oZ161 and oZ132 were used to amplify the 5' end of *ftsZ* in strains AK1 and AK18 as well as the wildtype control. Samples were analyzed via agarose gel electrophoresis. The additional bands on the gel are amplicons created by nonspecific binding of the primers. The lowest band in the AK18 lane is nonspecific and larger than the specific wildtype *ftsZ* bands in the wildtype and AK1 strains. B) Partial purification of α-FtsZ antibodies by exposing serum to a blot from a 12% SDS-PAGE gel fractionating extract from the *ftsZ*-null strain HU133. This western blot demonstrates nonspecific antibodies are removed from the serum. C) Whole cell extracts prepared from AK1 (*ftsZ*$^{+}$/*his*$_{8}$-*ftsZ*), AK18 (*his*$_{8}$-*ftsZ* /*his*$_{8}$-*ftsZ*), the wildtype strain M145, and the *ftsZ*-null strain HU133 (ΔftsZ::*aphI*) were fractionated on two identical 12% gels via SDS-PAGE. One gel was stained with Coomassie blue to be used as a loading control (left). The other gel was electrotransferred onto PVDF membrane for western blot analysis using partially purified α-FtsZ (right).

These *his*$_8$*-ftsZ* strains were further characterized using phase-contrast microscopy to look for sporulation. Wildtype *S. coelicolor* first grows as a branching network of vegetative mycelia within solid media. As the colony matures, aerial hyphae are produced that grow up and away from the medium. These aerial hyphae eventually undergo division to produce uninucleoid spores (Figure 8 - left). It has previously been shown that when *ftsZ* is deleted, the division required for sporulation is prevented (McCormick *et al*., 1994). Phase-contrast microscopy can be used to determine if His$_8$-FtsZ is functional by observing if these strains can sporulate. While the heterozygous strain was able to sporulate on SFM plates, the homozygous strain, expressing only

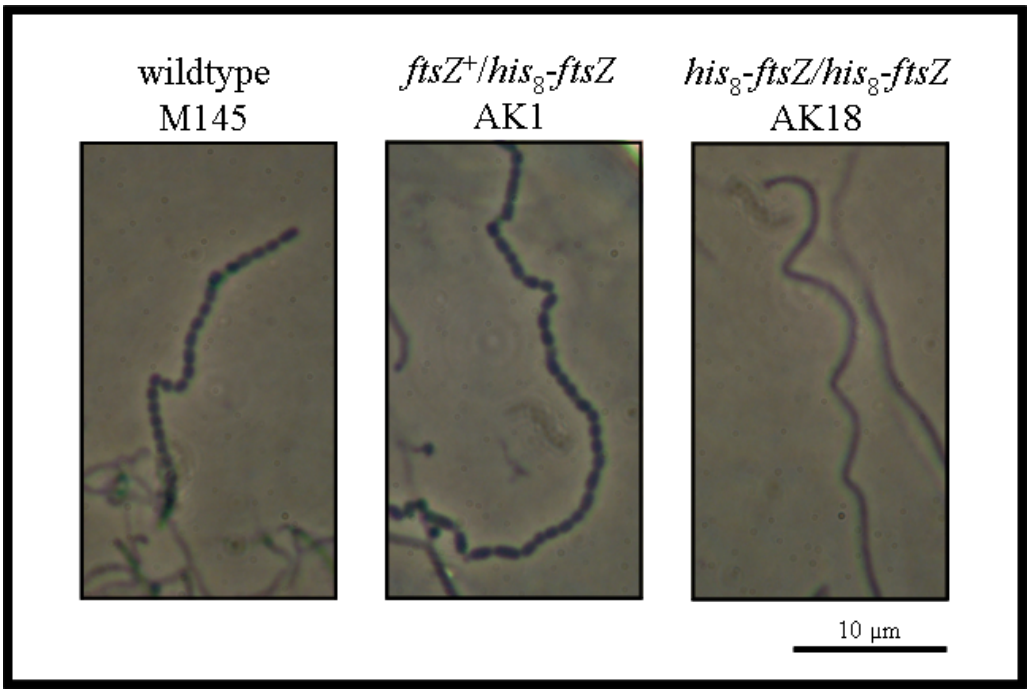

Figure 8

Phase-contrast microscopy of strains AK1 and AK18. The phenotypes of the two *S. coelicolor* strains expressing the epitope-tagged FtsZ were compared to the wildtype strain M145. While the heterozygous strain (AK1) was able to produce spores, the homozygous strain (AK18) was unable. All strains were grown on SFM agar for 5 days.

His_8-tagged FtsZ, appeared to be impaired and no spores were visible (Figure 8). Instead of observing a long chain of oval-shaped spores, long undivided aerial hyphae were observed. The *ftsZ*-null strain HU133 displayed a similar phenotype, although the aerial hyphae produced by HU133 did not coil (McCormick *et al*., 1994). This indicates that the fusion protein is not fully functional and that the epitope tag and/or scar peptide may be the cause of interference.

Construction and Analysis of *S. coelicolor* Strains that Express Epitope-Tagged FtsQ

Another cell division gene, *ftsQ*, was epitope-tagged utilizing the same procedure discussed above. In brief, modified pIJ773 disruption cassettes that were inserted into the 5' or 3' end of *ftsQ* were created via PCR. These cassettes were incorporated into the cosmid C69 via homologous recombination with the aid of the λ RED recombination plasmid pIJ790 (Table 7). FLP-mediated DNA recombination was utilized to remove the *aac(3)IV* selectable marker leaving an epitope tag sequence and an 81 bp *frt* site scar sequence (Table 8). The cosmids were made mobilizable by replacing the *bla* selectable marker on the backbone of the cosmids with the pIJ799 insertion-disruption cassette via λ RED-mediated recombination (Table 10). All cosmids were introduced into the chromosome of the wildtype *S. coelicolor* strain by homologous recombination following intraspecies conjugation.

Four different constructs were created and used to isolate four *S. coelicolor* strains, each of which expresses a different epitope-tagged FtsQ (Table 10). Two strains were designed to express His_8-epitope tag fusion proteins that can be utilized for a Ni-NTA pull down assay. One strain was tagged on the N-terminus and the other on the C-

terminus. The other two strains were designed to express HA epitope-tagged fusion proteins, one on the N-terminus and one on the C-terminus, which can be used to visualize FtsQ via western blot analysis. Epitope tag sequences were added to either end of the gene because one end of the encoded protein might be essential for function and the fusion may interfere with function or folding.

All transconjugants were analyzed via PCR (For an example see Figure S4) and four strains that were homozygous for the epitope-tagged gene were chosen to be studied: AK9 (*ftsQ-ha*/*ftsQ-ha*), AK10 (*ftsQ-his*$_8$/*ftsQ-his*$_8$), AK12 (*ha-ftsQ*/*ha-ftsQ*), and AK16 (*his*$_8$*-ftsQ*/*his*$_8$*-ftsQ*). Before these strains were studied further, the additional copy of the *dcw* gene cluster was removed by growing each strain on SFM agar without antibiotic selection. This encouraged an intramolecular homologous recombination event between the ~35 kb duplicated regions of the chromosome (Figure 9). Strains that have undergone this "loop-out" event were identified based upon sensitivity to apramycin and kanamycin, the two antibiotic-resistance genes found in the back bone of the cosmid that had previously incorporated into the chromosome. Three new strains were isolated by this process: AK13 (*ha-ftsQ*), AK14 (*ftsQ-ha*), and AK15 (*ftsQ-his*$_8$). A strain that had undergone intramolecular recombination was not isolated from AK16 at this time; therefore, the merodiploid strain AK16 was further analyzed with the three completed strains. All of these strains were verified via PCR to ensure that the epitope tag sequence was still fused to *ftsQ* (Figure 10). An FtsQ antiserum was obtained (Stadelmaier B, Kuennen R, and McCormick JR, unpublished data) and an attempt to visualize fusion proteins via western blot analysis using these antibodies was performed. The blot was deemed inconclusive, however, due to a high background of nonspecific binding in the

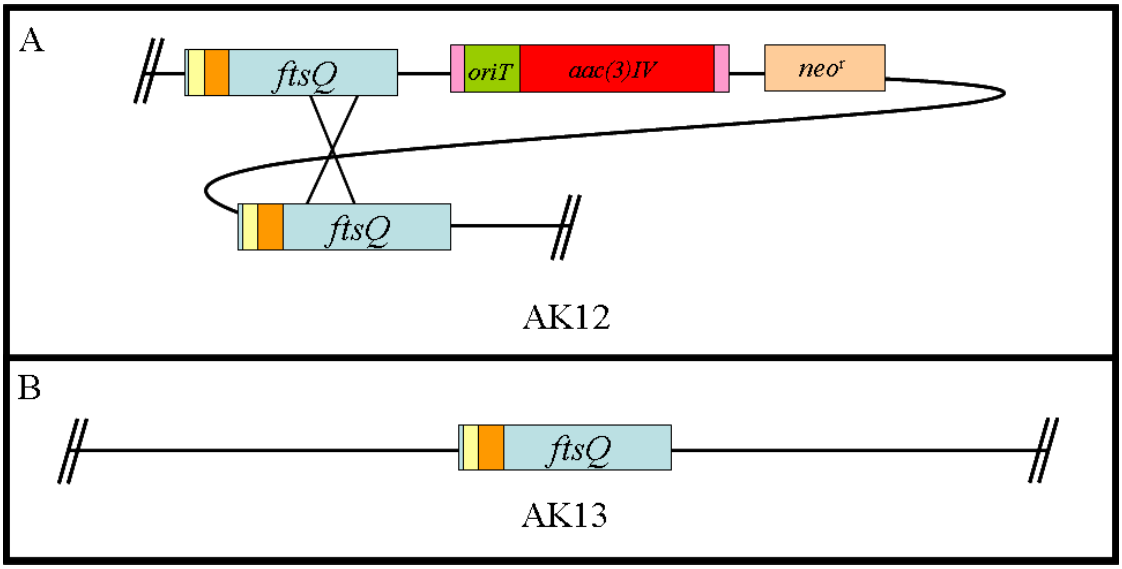

Figure 9

Intramolecular homologous recombination between duplicated regions of the *S. coelicolor* chromosome. To remove the duplicate copy of the *dcw* gene cluster strains were grown on SFM agar without antibiotic selection. A) In this example, the merodiploid strain AK12 underwent an intramolecular homologous recombination event. For simplicity sake, the homologous recombination event is pictured happening between two copies of *ha-ftsQ*. This event could actually take place between any regions of the duplicated *dcw* gene cluster. B) The resulting strain, AK13, was isolated based on apramycin- and kanamycin-sensitivity, the markers found in the cosmid backbone. This strain contains one copy of the *dcw* gene cluster and expresses HA-FtsQ. The yellow boxes represent the *ha* epitope tag sequence and the orange boxes represents the 81 bp *frt* site scar sequence. The *neo*^{r} gene confers resistance to kanamycin.

α-FtsQ antiserum and possibly because FtsQ was not present at high enough levels in extracts from liquid-grown cultures (Figure S5).

The division phenotypes of these strains were studied by phase-contrast microscopy using the sporulation assay. All strains were compared to the wildtype strain M145 to determine if epitope-tagging FtsQ had an effect on sporulation. It was previously found that the *ftsQ*-null strain, HU151, had a phenotype similar to that of the *ftsZ*-null strain HU133 (McCormick and Losick, 1996). While epitope-tagging FtsZ prevented sporulation, this effect was not found when FtsQ was epitope-tagged. All

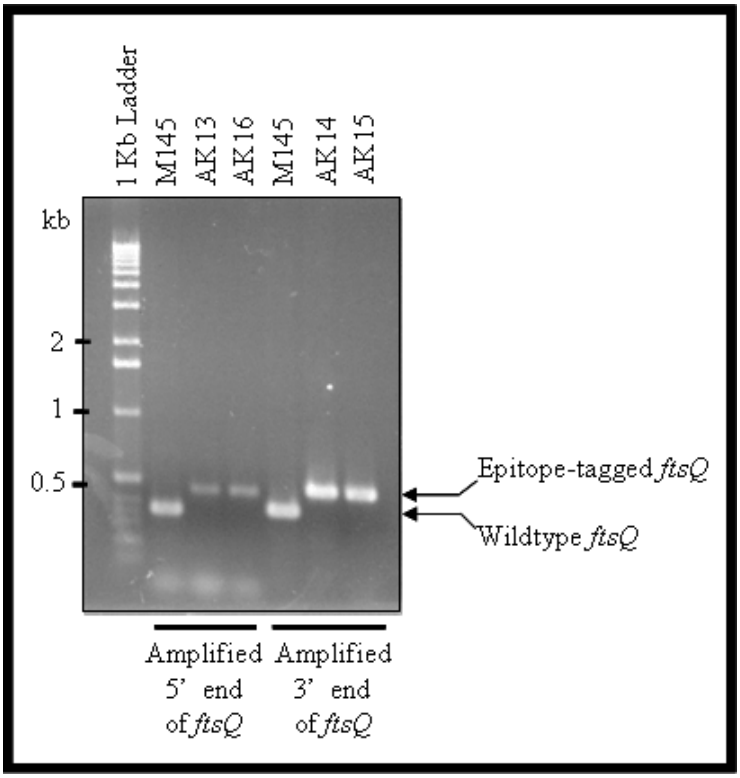

Figure 10

PCR analysis of *S. coelicolor* strains that express epitope-tagged FtsQ. Primers oQ160 and oQ161 were used to amplify the 5' end of *ftsQ* in strains AK13 (*ha-ftsQ*) and AK16 (his_8-*ftsQ*/his_8-*ftsQ*). Primers oQ174 and oQ165 were used to amplify the 3' end of *ftsQ* in strains AK14 (*ftsQ-ha*) and AK15(*ftsQ*-his_8) All samples were analyzed via gel electrophoresis and compared to the wildtype strain M145.

strains were able to sporulate efficiently, although irregularly shaped spores were occasionally found (Figure 11). These results indicate that epitope-tagging FtsQ produces fusion proteins that are functional, because these strains do not behave as the *ftsQ*-null strain, which is unable to sporulate efficiently (McCormick and Losick, 1996).

A Southern blot hybridization analysis was performed on all "loop-out" strains to ensure that deletions or rearrangements of the chromosome had not occurred during the two previous homologous recombination events. Chromosomal DNA from the wildtype strain M145, AK13 (*ha-ftsQ*), AK14 (*ftsQ-ha*), and AK15 (*ftsQ*-his_8) were digested

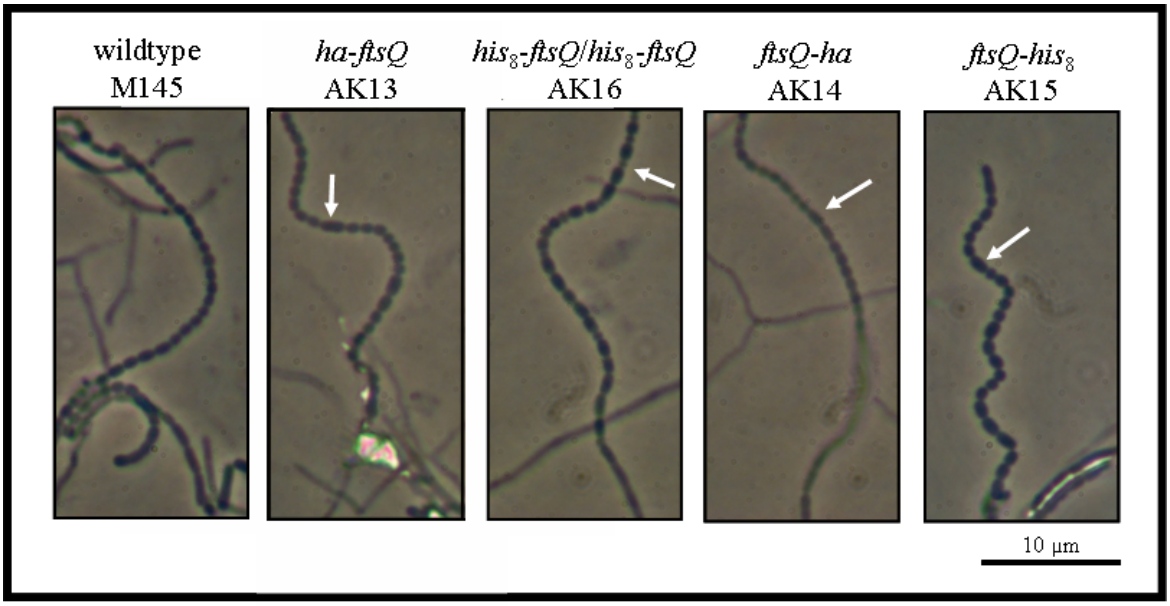

Figure 11

Phase-contrast microscopy of *S. coelicolor* strains that express epitope-tagged FtsQ. The phenotypes of the four *S. coelicolor* strains expressing the epitope-tagged FtsQ were compared to the wildtype strain M145. Each of these strains was able to produce spores, although some spores were found to be irregularly shaped (arrows). All strains were grown on SFM agar for 5 days.

independently with *Sac*I, *Xho*I, and *Pst*I. The DNA was fractionated via gel electrophoresis, blotted to a neutral nylon membrane, and probed with DNA fragments from the *Xho*I-digested cosmid C69. The *Sac*I and *Xho*I digests of the wildtype strain and the strains the express epitope-tagged FtsQ should look similar because no additional restriction sites for these enzymes are found in either epitope tag sequence or the *frt* site scar sequence (Figure 12A). The only difference between these strains is an additional 105 bp (caused by the 24 bp his_8 epitope sequence and the 81 bp *frt* site scar sequence) or 108 bp (caused by the 27 bp *ha* epitope tag sequence and the 81 bp *frt* site scar sequence). Such a small increase in band size will not be noticed via 0.8% agarose gel electrophoresis. The *frt* site scar sequence, however, adds an additional *Pst*I site in the

strains that express epitope-tagged FtsQ, which will cause the 21.8 kb fragment found in the wildtype strain to be cleaved in two. In AK13 (*ha-ftsQ*), this additional cleavage produces a 14.2 kb and a 7.6 kb fragment (Figure 12B). In AK14 (*ftsQ-ha*) and AK15 (*ftsQ-his*$_8$) this additional cleavage produces a 15 kb and a 6.9 kb fragment (Figure 12B). The predicted bands of the *Sac*I, *Xho*I, and *Pst*I digests were detected, indicating that no major deletions or rearrangement of the chromosome have occurred in the "loop-out" strains that express epitope-tagged FtsQ when introducing the gene fusions into the chromosome.

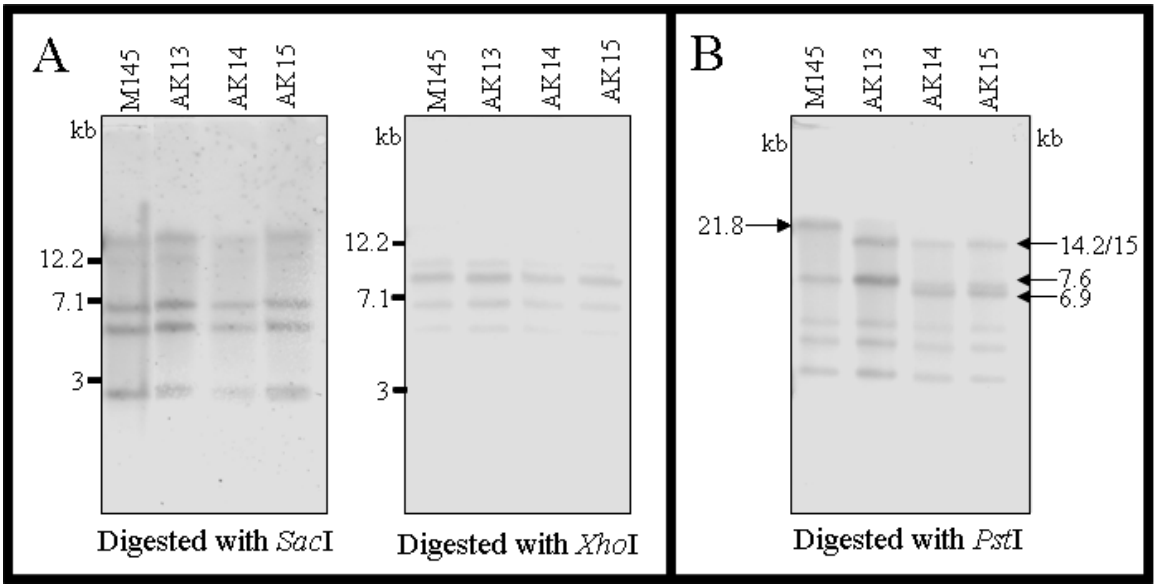

Figure 12

Southern blot hybridization analysis of the *S. coelicolor* strains AK13, AK14, and AK15. A) The wildtype strain M145 and all other strains were digested with *Sac*I and *Xho*I, fractionated via gel electrophoresis, and blotted to a membrane. Detection of bands was carried out by using *Xho*I-digested C69 as a probe. No difference is seen in the banding pattern of all strains. B) M145 and all other strains were digested with *Pst*I, fractionated, blotted, and detected as before. A 21.8 kb fragment found in M145 is seen as two separate bands, 14.2 kb and 7.6 kb, in AK13 (*ha-ftsQ*). In AK14 (*ftsQ-ha*) and AK15 (*ftsQ-his*$_8$), this 21.8 kb fragment is cleaved into a 15 kb and a 6.9 kb fragment.

Construction and Analysis of *S. coelicolor* Strains that Express Epitope-Tagged FtsW

The cell division gene *ftsW* was epitope-tagged next. As discussed above for FtsQ, four different constructs were created that would be used to isolate these strains (Table 10). These constructs were used to isolate two strains that would express His_8-tagged FtsW and two that would express HA-tagged FtsW. Again, both the N-terminus and the C-terminus of FtsW were tagged. If one end of FtsW was found to be required for function or folding, then a strain with an epitope tag on the opposite end might be functional.

Like the epitope-tagged *ftsQ* strains, all transconjugants were analyzed via PCR (For an example see Figure S4) and four were chosen for further study: AK2 (*his*$_8$-*ftsW*/*his*$_8$-*ftsW*), AK6 (*ha-ftsW*/*ha-ftsW*), AK7 (*ftsW*$^+$/*ftsW-ha*), and AK23 (*ftsW-his*$_8$/*ftsW-his*$_8$). The additional copy of the *dcw* gene cluster was removed from the strains AK2 and AK6 via spontaneous intramolecular homologous recombination resulting in strains AK4 (*his*$_8$-*ftsW*) and AK20 (*ha-ftsW*), respectively. These new strains were analyzed with the merodiploid strains AK7 and AK23 via PCR analysis (Figure 13).

The fusion proteins expressed from the constructed genes could not be visualized via western blot analysis due to the fact that polyclonal antibodies were never raised against FtsW. Alternatively, antibodies specific for the epitope tag can be used. Attempts to visualized His_8-FtsW and FtsW-His_8 using α-His antibodies has not worked thus far (Figure S6).

Phase-contrast microscopy was again used to study strains expressing FtsW fusion proteins. It was previously found that the *ftsW*-null strain PFB26 had the rich medium-dependent phenotype of sporulation inhibition (Bidey, 2004). Instead of

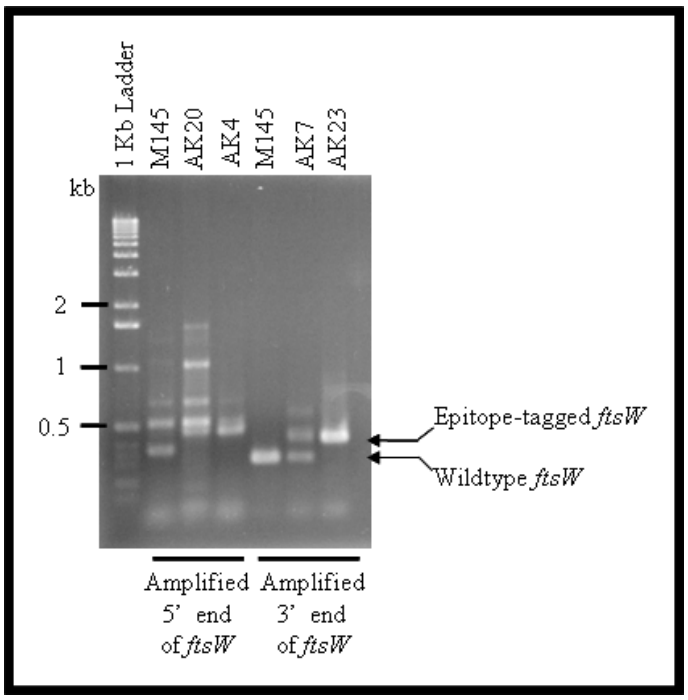

Figure 13

PCR analysis of *S. coelicolor* strains that express epitope-tagged FtsW. Primers oW164 and oW170 were used to amplify the 5' end of *ftsW* in strains AK20 (*ha-ftsW*) and AK4 (his_8-*ftsW*). Primers oW119 and oW156 were used to amplify the 3' end of *ftsW* in strains AK7 ($ftsW^+$/*ftsW-ha*) and AK23 (*ftsW-*his_8/*ftsW-*his_8). All samples were then analyzed via gel electrophoresis and compared to the wildtype strain M145. Additional bands on the gel are amplicons created by nonspecific binding of the primers used.

producing normal spore chains, the *ftsW*-null strain produced short, thick, undifferentiated aerial filaments when grown on this medium (Figure 14). However, this strain was able to sporulate, albeit poorly, when grown on minimal media (MM) (Figure 14). To test if these FtsW fusion proteins were functional, all strains were grown on R2YE and MM agar and compared to the wildtype strain M145 and the *ftsW*-null strain PFB26 (Figure 15). None of the newly created strains were able to sporulate properly on R2YE, which suggests that the fusion proteins are not functional because these strains possess a phenotype similar strains possess a phenotype similar to that of the *ftsW*-null

strain. Interestingly, spore chains were not found when AK7 (*ftsW*$^{+}$/*ftsW-ha*) and AK23 (*ftsW-his*$_8$/*ftsW-his*$_8$) were grown on MM either, where the effect in the *ftsW*-null strain is suppressed (Figure 15B). This may indicate a dominant negative phenotype when the 5' end of *ftsW* is epitope-tagged.

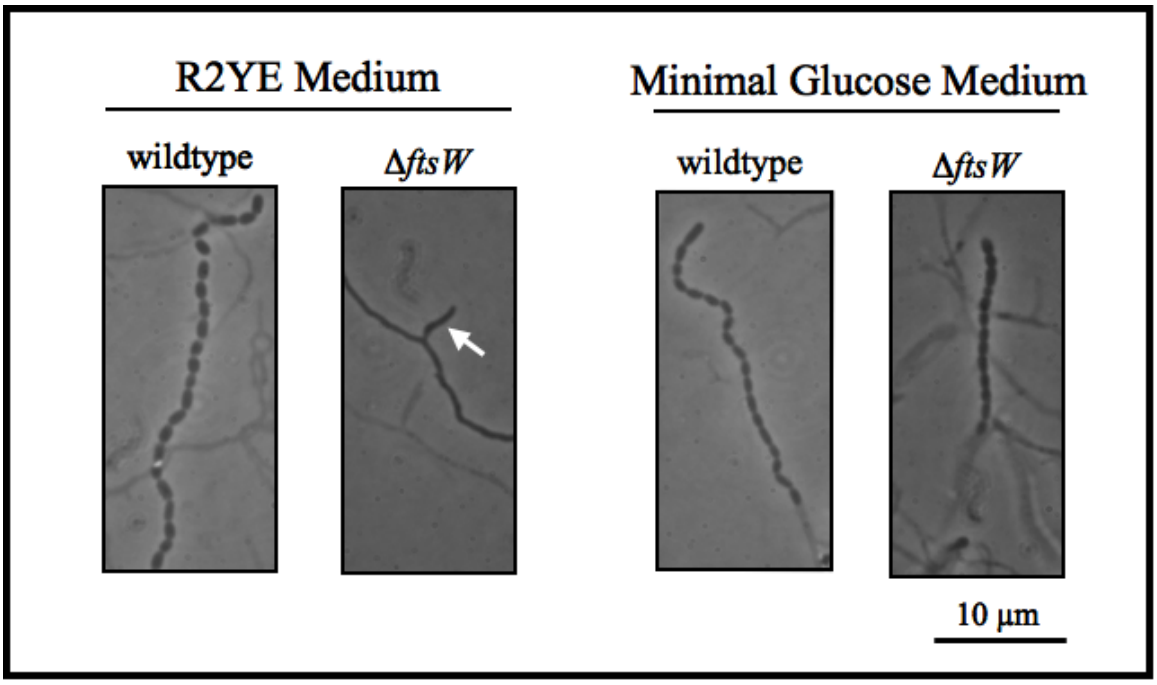

Figure 14

Phase-contrast microscopy of the Δ*ftsW S. coelicolor* strain. The phenotypes of the wildtype strain M145 and the *ftsW*-null strain PFB26 were compared by phase-contrast microscopy. The wildtype strain is able to sporulate on R2YE and MM. The *ftsW*-null strain cannot sporulate on R2YE. Instead, short, undifferentiated aerial hyphae are observed (arrow). This strain can sportulate, albeit poorly, on MM. All strains were grown on R2YE and MM agar for 5 days.

A Southern blot hybridization analysis was performed on AK4 (*his*$_8$-*ftsW*) and AK20 (*ha-ftsW*) to ensure that deletions or rearrangements of the chromosome had not occurred during the two previous homologous recombination events. Chromosomal

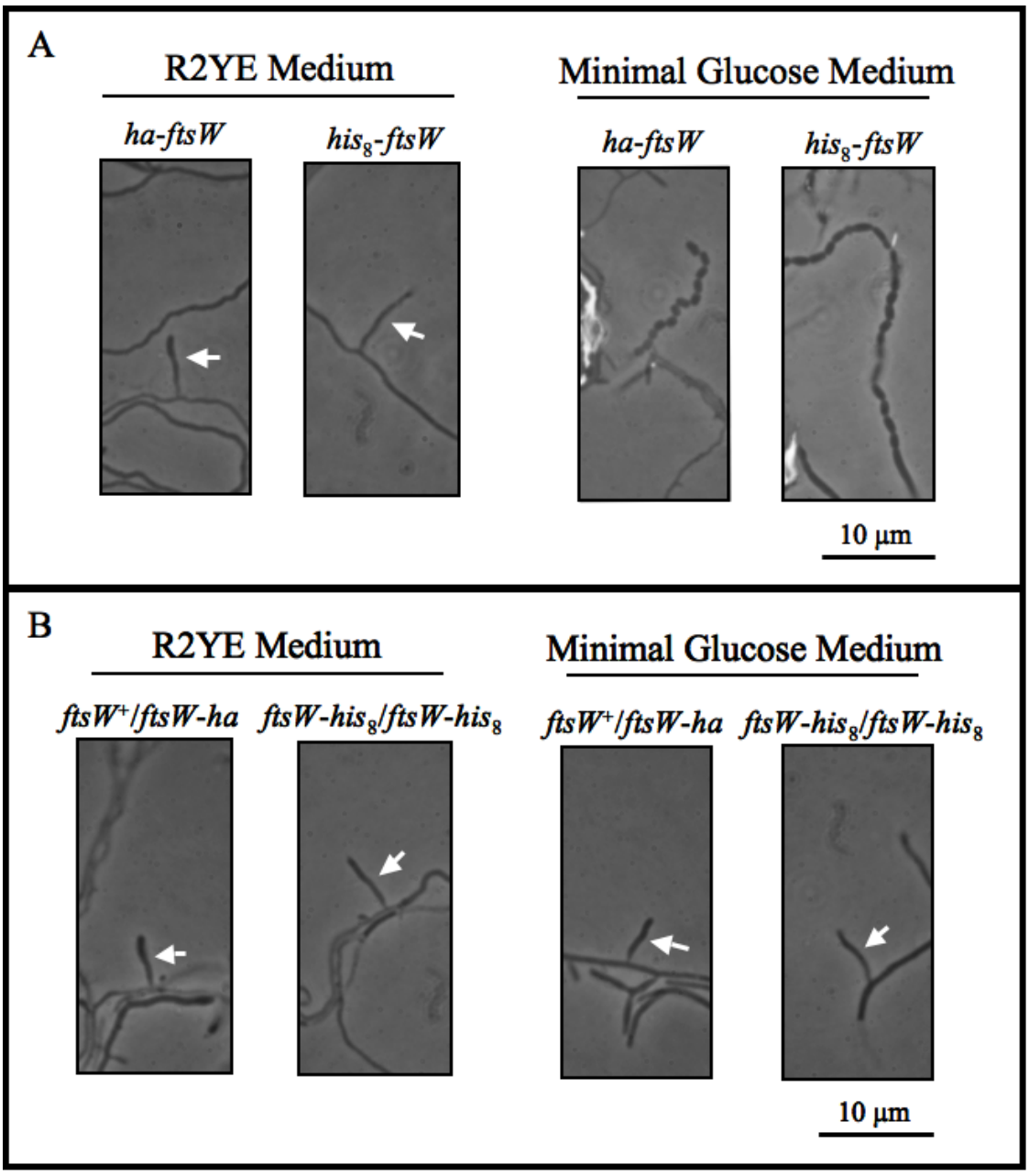

Figure 15

Phase-contrast microscopy of *S. coelicolor* strains that express epitope-tagged FtsW. The phenotypes of the four *S. coelicolor* strains expressing the epitope-tagged FtsW were compared to the wildtype strain M145 and the *ftsW*-null strain PFB26. A) Strains expressing HA- and His_8-epitope tags on the 5' end of *ftsW* display the same phenotype as the *ftsW*-null strain indicating that these fusion proteins are not functional. B) Strains expressing Ha- and His_8-epitope tags on the 3' end of *ftsW* display a dominate negative phenotype and are unable to sporulate on R2YE and MM. White arrows indicate short, undifferentiated aerial hyphae. All strains were grown on R2YE and MM agar for 5 days.

DNA from the wildtype strain M145, AK4, and AK20 were digested independently with *Sac*I, *Xho*I, and *Pst*I. The DNA was fractionated via gel electrophoresis and blotted to a neutral nylon membrane. The blot was probed with DNA fragments from the *Xho*I-digested cosmid C69. The *Sac*I and *Xho*I digests of M145 and AK4 should look similar because no additional restriction sites for these enzymes are found in the epitope tag sequences of AK4 and AK20 or the *frt* site sequence (Figure 16A). The only difference between these strains is an additional 105 bp (caused by the 24 bp his_8 epitope sequence and the 81 bp *frt* site scar sequence) or 108 bp (caused by the 27 bp *ha* epitope tag sequence and the 81 bp *frt* site scar sequence). Such a small increase in band size will

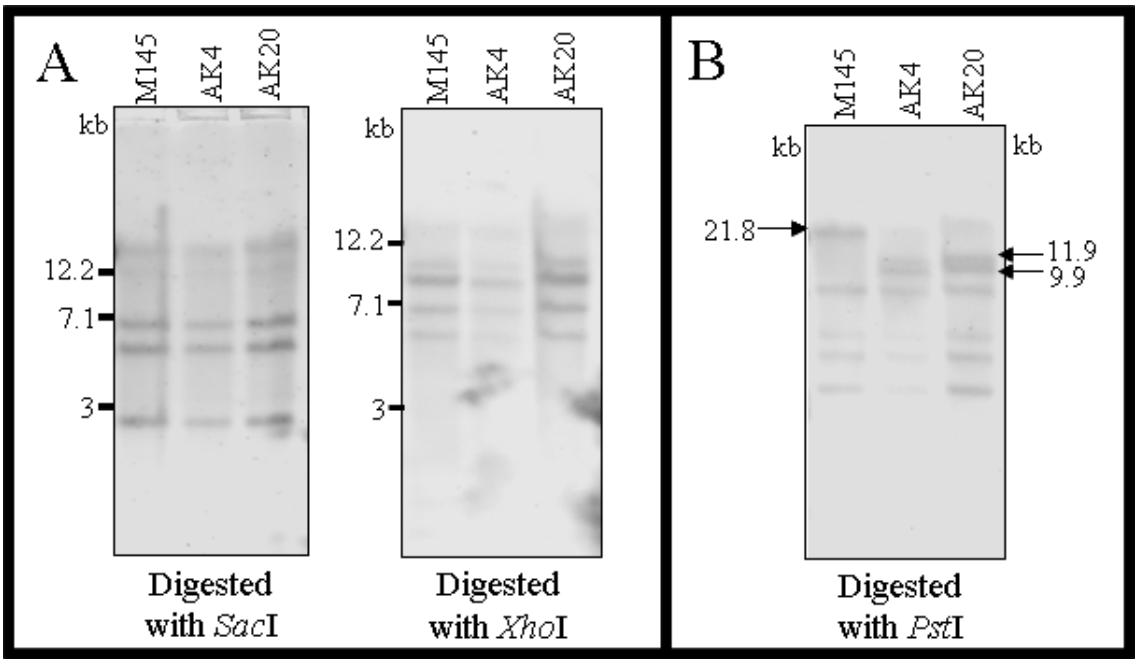

Figure 16

Southern blot hybridization analysis of the *S. coelicolor* strains AK4 and AK20. A) The wildtype strain M145, AK4 (his_8-*ftsW*), and AK20 (*ha*-*ftsW*) were digested with *Sac*I and *Xho*I, fractionated via gel electrophoresis, and blotted to a membrane. Detection of bands was carried out by using *Xho*I-digested C69 as a probe. No difference is seen in the banding pattern of all strains. B) M145, AK4, and AK20 were digested with *Pst*I, fractionated, blotted, and detected as before.

not be noticed via 0.8% agarose gel electrophoresis. The *frt* site scar sequence, however, adds an additional *Pst*I site in AK4 and AK20, which causes the 21.8 kb fragment found in the wildtype strain to be cleaved into an 11.9 kb and a 9.9 kb fragment in both strains (Figure 16B). The predicted bands from the *Sac*I, *Xho*I, and *Pst*I digests were detected, indicating that no major deletions or rearrangement of the chromosome have occurred in AK4 or AK20 when introducing *his*$_8$-*ftsW* and *ha-ftsW* into the chromosome.

Construction and Analysis of *S. coelicolor* Strains that Express Epitope-Tagged FtsI

The last cell division gene to be epitope-tagged in this study was *ftsI*. Four constructs were created to make these strains as well (Table 10). However, only the C-terminus of this protein was tagged. Constructs created to epitope-tag the N-terminus could not be verified via PCR analysis because the oligonucleotides oI152 and oI167, which were designed to amplify the 3' end of *ftsI*, did not amplify the correct region of DNA. Otherwise, these strains were created in the same fashion as the epitope-tagged FtsQ and FtsW strains. Two transconjugants were selected based upon PCR analysis: AK19 (*ftsI-ha*/*ftsI-ha*) and AK22 (*ftsI*$^+$/*ftsI-his*$_8$) (Figure 17). The merodiploid strains were analyzed because no strains that had undergone intramolecular homologous recombination to remove the additional *dcw* gene cluster have been isolated to date. An attempt to visualize fusion proteins via western blot analysis failed due to the high background of nonspecific binding of the α-FtsI antiserum and possibly because FtsI was not present at high enough levels in extracts prepared from liquid-grown cultures (Figure S7).

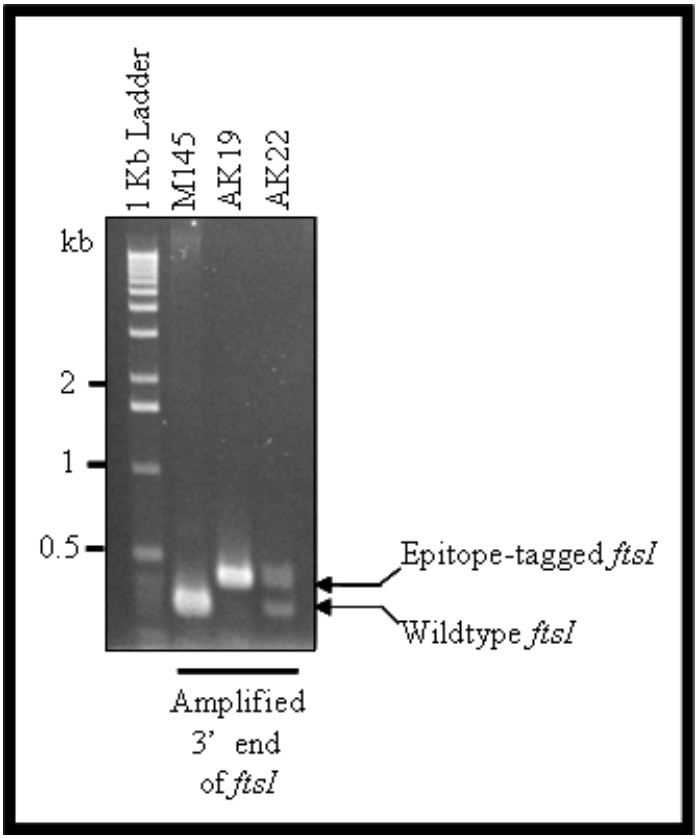

Figure 17

PCR analysis of *S. coelicolor* strains that express C-terminal epitope-tagged FtsI. Primers oI144 and oI139 were used to amplify the 3' end of *ftsI* in strains AK19 (*ftsI-ha*/*ftsI-ha*) and AK22 ($ftsI^{+}$/*ftsI-his*$_8$). All samples were then analyzed via gel electrophoresis and compared to the wildtype strain M145.

To determine if the fusion proteins being expressed in these strains were functional, phase-contrast microscopy was again used to see if sporulation was occurring normally. It was previously found that the *ftsI*-null strain JBY5 had the same medium-dependent phenotype as the *ftsW*-null strain (Figure 18) (Bennett, 2006). Therefore, to test if the FtsI fusion proteins were functional, all strains were grown on R2YE and MM agar and compared to the wildtype strain M145 and the *ftsI*-null strain JBY5 (Figure 19). AK22 ($ftsI^{+}$/*ftsI-his*$_8$) behaved like the *ftsI*-null strain, not sporulating on R2YE and

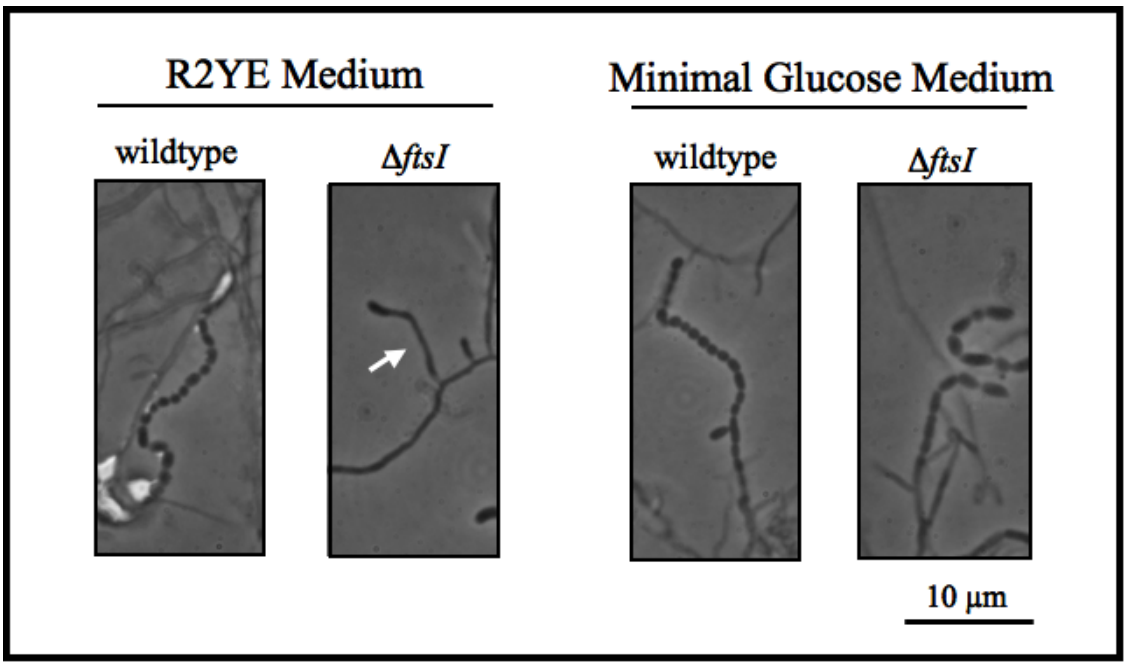

Figure 18

Phase-contrast microscopy of the Δ*ftsI S. coelicolor* strain. The phenotypes of the wildtype strain M145 and the *ftsI*-null strain JBY5 were compared by phase-contrast microscopy. The wildtype strain is able to sporulate on R2YE and MM. The *ftsI*-null strain cannot sporulate on R2YE. Instead, short, undifferentiated aerial hyphae are observed (arrow). This strain can sportulate, albeit poorly, on MM. All strains were grown on R2YE and MM agar for 5 days.

sporulating on MM, suggesting that the fusion protein is not functional and that the tagged gene was dominant to wildtype. AK19 (*ftsI-ha*/*ftsI-ha*), however, was found to sporulate on R2YE, which would suggest that this fusion is functional. If the FRT scar peptide prevented function, it would be expected that these two strains should behave in a similar manner since they both express fusion proteins that contain epitope tags at the C-terminus of FtsI. Therefore, it appears that the His_8 epitope tag inhibits function of FtsI while the HA epitope tag does not. Table 13 summarizes the data for all fusion proteins analyzed in this study.

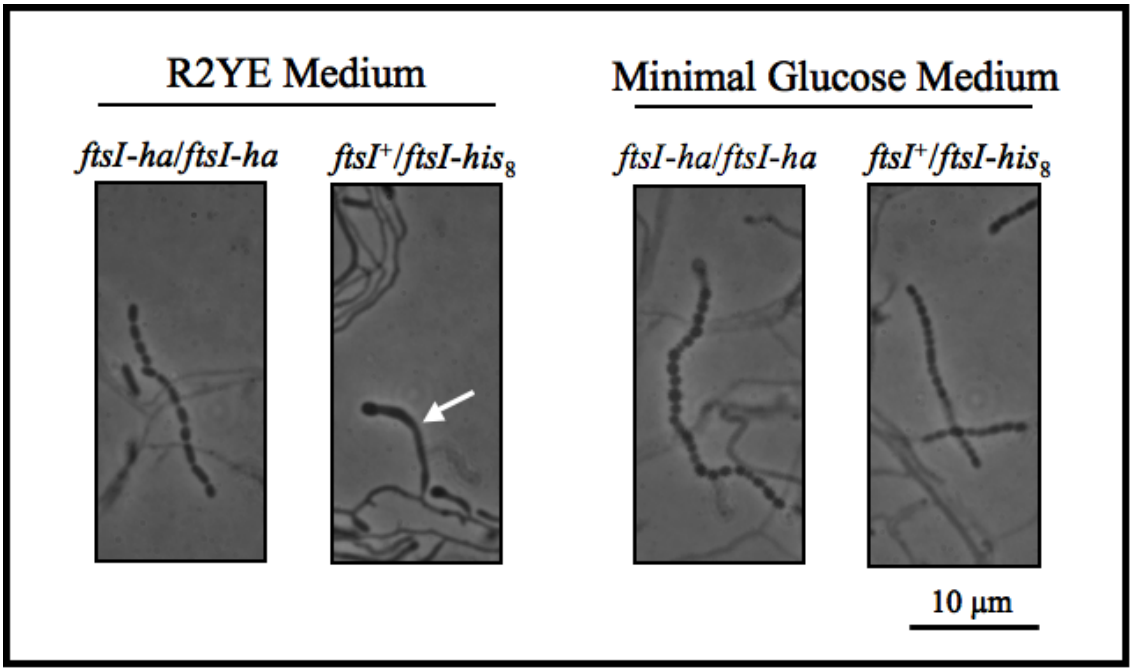

Figure 19

Phase-contrast microscopy of *S. coelicolor* strains that express C-terminal epitope-tagged FtsI. The phenotypes of the two *S. coelicolor* strains expressing the epitope-tagged FtsI were compared to the wildtype strain M145 and the *ftsI*-null strain JBY5 (Δ*ftsI*::*aphI*). AK19 was found to sporulate on R2YE. AK22 behaved like JBY5, and produced short, thick, undifferentiated aerial hyphae on R2YE (arrow). All strains were able to sporulate on MM, although many more spores were found to be irregularly shaped in the strains JBY5, AK19, and AK22 than in M145. All strains were grown on R2YE and MM agar for 5 days.

Table 13

Summary of the Analysis of Epitope-Tagged Cell Division Proteins

Strain	Genotype	Medium	Sporulation	Functional
AK1	*ftsZ*$^{+}$/*his*$_{8}$-*ftsZ*	SFM	Yes	Partially
AK18	*his*$_{8}$-*ftsZ*/*his*$_{8}$-*ftsZ*	SFM	No	Partially
AK13	*ha-ftsQ*	SFM	Yes	Yes
AK16	*his*$_{8}$-*ftsQ*/*his*$_{8}$-*ftsQ*	SFM	Yes	Yes

Table 13 (continued)

Strain	Genotype	Medium		Sporulation		Functional
AK14	*ftsQ-ha*	SFM		Yes		Yes
AK15	*ftsQ-his*$_8$	SFM		Yes		Yes
AK20	*ha-ftsW*	R2YE	MM	No	Yes	No
AK4	*his*$_8$*-ftsW*	R2YE	MM	No	Yes	No
AK7	*ftsW*$^+$/*ftsW-ha*	R2YE	MM	No	No	No
AK23	*ftsW-his*$_8$/*ftsW-his*$_8$	R2YE	MM	No	No	No
AK19	*ftsI-ha*/*ftsI-ha*	R2YE	MM	Yes	Yes	Yes
AK22	*ftsI*$^+$/*ftsW-his*$_8$	R2YE	MM	No	No	No

Using a Ni-NTA Pull Down Assay to Study Protein-Protein Interactions

The strains described above were created so that protein-protein interactions between cell division proteins in *S. coelicolor* could be studied. The idea was that His_8-tagged proteins could be isolated by using a Ni-NTA pull down assay and interacting proteins could be visualized via western blot analysis using α-FtsZ, α-FtsQ, α-FtsI, or α-HA antibodies. The first step in adapting this assay was to determine if His_8 epitope-tagged proteins present in a whole cell extract could bind to the Ni-NTA resin and that these proteins could be eluted by competitive displacement with imidazole. The strain AK1 was used to test this assay because FtsZ is the only cell division protein in this study that can be readily and reproducibly visualized via western blot analysis at this time and because AK1 expresses both FtsZ and His_8-FtsZ. The soluble and crude membrane fractions of AK1 were isolated. The membrane fraction was solublized in native

conditions using Nonidet P-40 (NP-40). Solublized proteins were incubated with Ni-NTA resin. The resin was washed with solubilizing buffer to remove the bulk of unbound protein and with a low salt buffer (0.5 M NaCl) to remove any loosely associated proteins. Next, the resin was washed with a buffer containing imidazole (500 mM) to remove His_8-FtsZ. Lastly, the resin was boiled to remove any tightly bound protein. All washes were collected, TCA precipitated, fractionated via SDS-PAGE, and analyzed by western blot analysis (Figure 20). Preliminary results indicate that this pull down assay works. His_8-FtsZ bound to the resin, while authentic FtsZ did not. The majority of the fusion protein eluted with 100 mM imidazole. Some remained tightly bound to the resin and was removed when the resin was boiled in SDS buffer.

Since the Ni-NTA pull down showed binding of the His_8-FtsZ, this technique was used to determine if FtsW and FtsI directly interact. This interaction was proposed in *E. coli* and *B. subtilis* (Di Lallo *et al*., 2003; Karimova *et al*., 2005; Daniel *et al*., 2006). The solubilized membrane fraction from the *S. coelicolor* strain that expresses His_8-FtsW (AK4) was used. Samples were analyzed via SDS-PAGE and western blot analysis using α-FtsI to see if FtsI was pulled down with His_8-FtsW. More washes were performed during this experiment to remove as much contaminating protein as possible from the resin since the α-FtsI antibodies have been found to bind nonspecifically to many proteins. The wildtype strain M145 was analyzed as a negative control. A commercially available α-His antibody (Santa Cruz) was used in a similar pull down experiment to visualize His_8-FtsW, however no bands were visualized (Figure S6). Another α-His antibody (Qiagen) was tested and yielded similar unsatisfactory results (Figure S8). When α-FtsI antibodies were used, many nonspecific bands appeared in all lanes

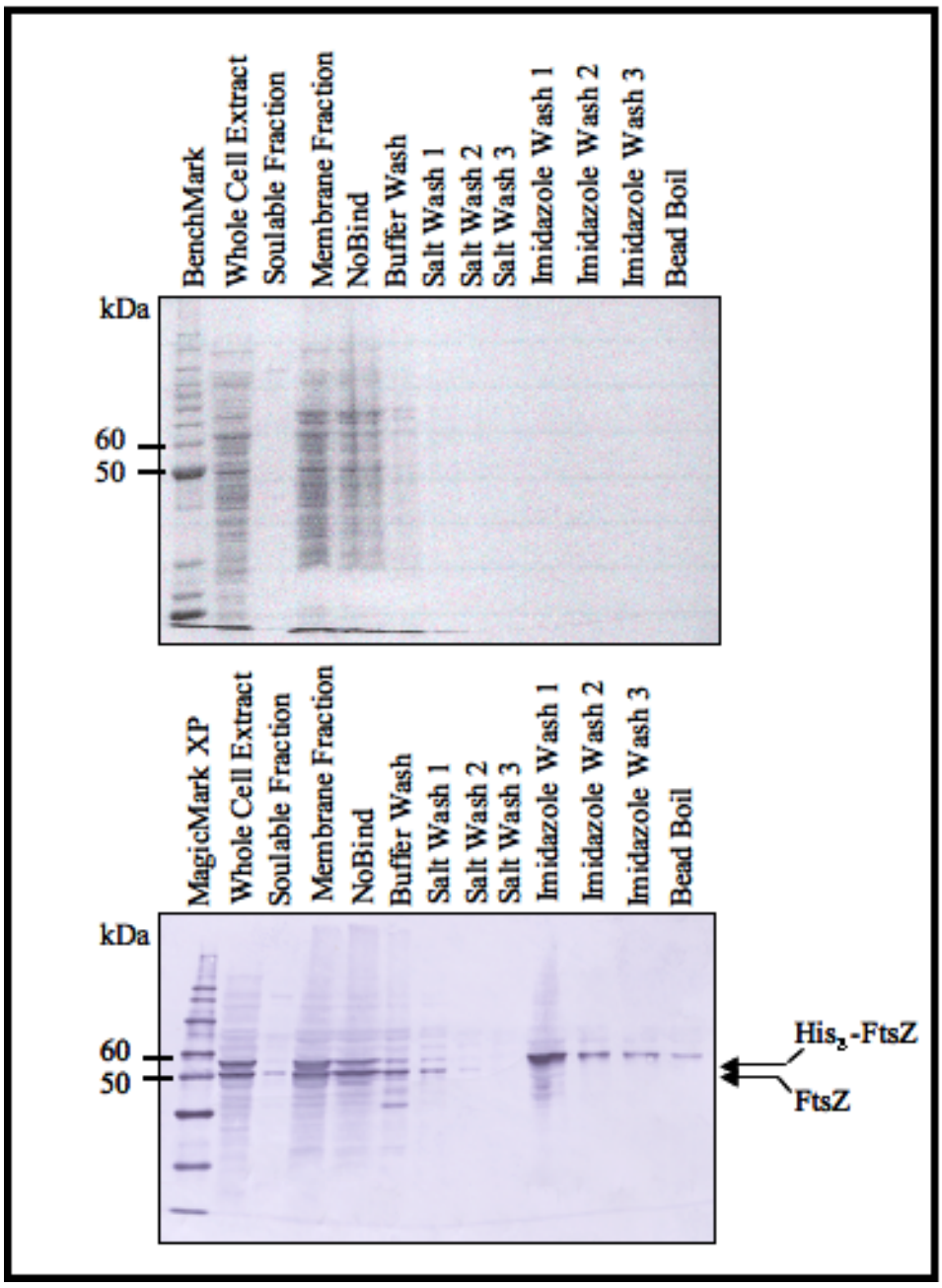

Figure 20

Western blot analysis of the Ni-NTA pull down assay using the solubilized membrane fraction of AK1. A Ni-NTA pull down was performed and all samples were TCA precipitated, fractionated via SDS-PAGE on a 10% gel (top), and analyzed by western blot analysis using α-FtsZ (bottom). While His_8-FtsZ binds the resin and elutes when the resin is washed with imidazole, FtsZ never binds. Each salt wash contained 0.5 M NaCl. Each imidazole wash contained 500 mM imidazole.

(Figure 21). No different between the wildtype extract and the extract containing His_8-FtsW was observed. These results indicate antibodies specific for His- and HA-epitope tags need to be acquired and that the α-FtsI antibodies need to be affinity purified.

Since the α-FtsI antibodies being used in these western blot experiments were not working well, another approach to visualize the results for the Ni-NTA pull down assay was explored. Instead of performing a western blot analysis on pull down fractions, gels were stained using a nickel-staining kit (Fisher Scientific). This stain is about ten times more sensitive than the more commonly used Coomassie blue stain. A typical Ni-NTA pull down was performed using the solubilized membrane fraction of AK4 (*his_8-ftsW*). More buffer, salt, and imidazole washes were carried out to remove as much nonspecific protein from the resin as possible, in as many fractions as possible, so that lanes would not be overloaded. After fractionating the samples via SDS-PAGE, the early fractions and washes were stained using Coomassie blue stain and the later washes were nickel-stained (Figure 22A). Five distinct bands are seen in the first 100 mM imidazole wash, which indicate that these proteins have either bound to the resin itself or to His_8-FtsW. As a negative control, the same experiment was performed using the solubilized membrane fraction of the wildtype strain M145 (Figure 22B). The same five bands are also found in the first 100 mM imidazole wash, which indicates that nonspecific proteins are binding the Ni-NTA resin. As shown above, the epitope-tagged FtsW fusion proteins created in this study are not functional (Figure 15A). This could account for why additional proteins were not found in the first 100 mM imidazole wash. The fusion protein may be unstable and therefore may not accumulate. As mentioned previously, FtsW may not be expressed at high enough levels when strains are grown in liquid

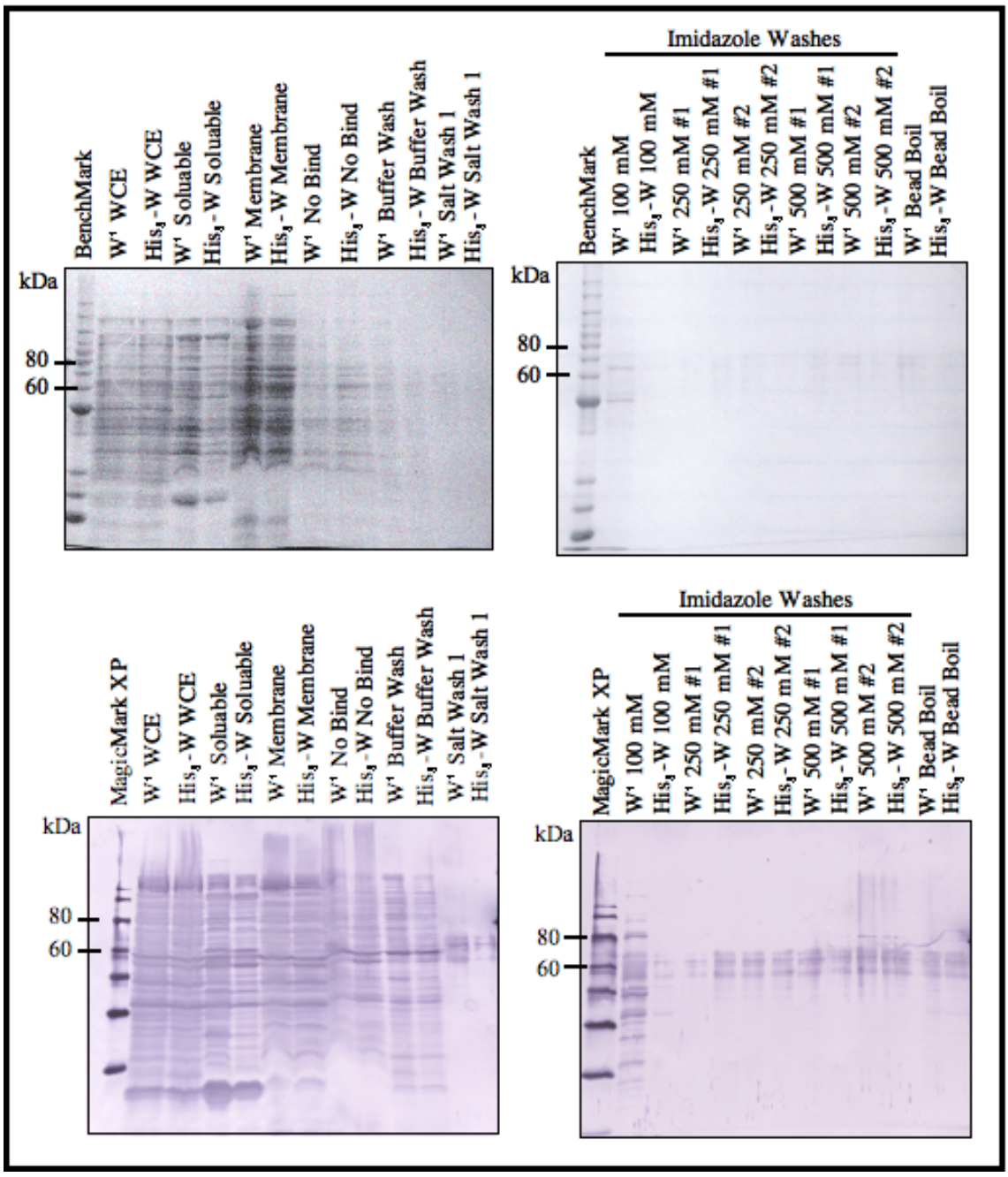

Figure 21

Western blot analyses of Ni-NTA pull down assays using whole cell extract from a strain expressing His_8-FtsW. Pull down assays were performed using extract from the strain expressed His_8-FtsW (His_8-W). Fractions were isolated and fractionated on a 10% SDS-PAGE gel (top). A western blot analysis were performed with α-FtsI primary antibodies (bottom). The left blot contains early washes. If FtsI interacts with FtsW, it would be expected to elute with His_8-FtsI during the imidazole washes (bottom right). M145 fractions were used as a negative control (W^+). The predicted molecular weight of FtsI is 69 kDa. (WCE - whole cell extract)

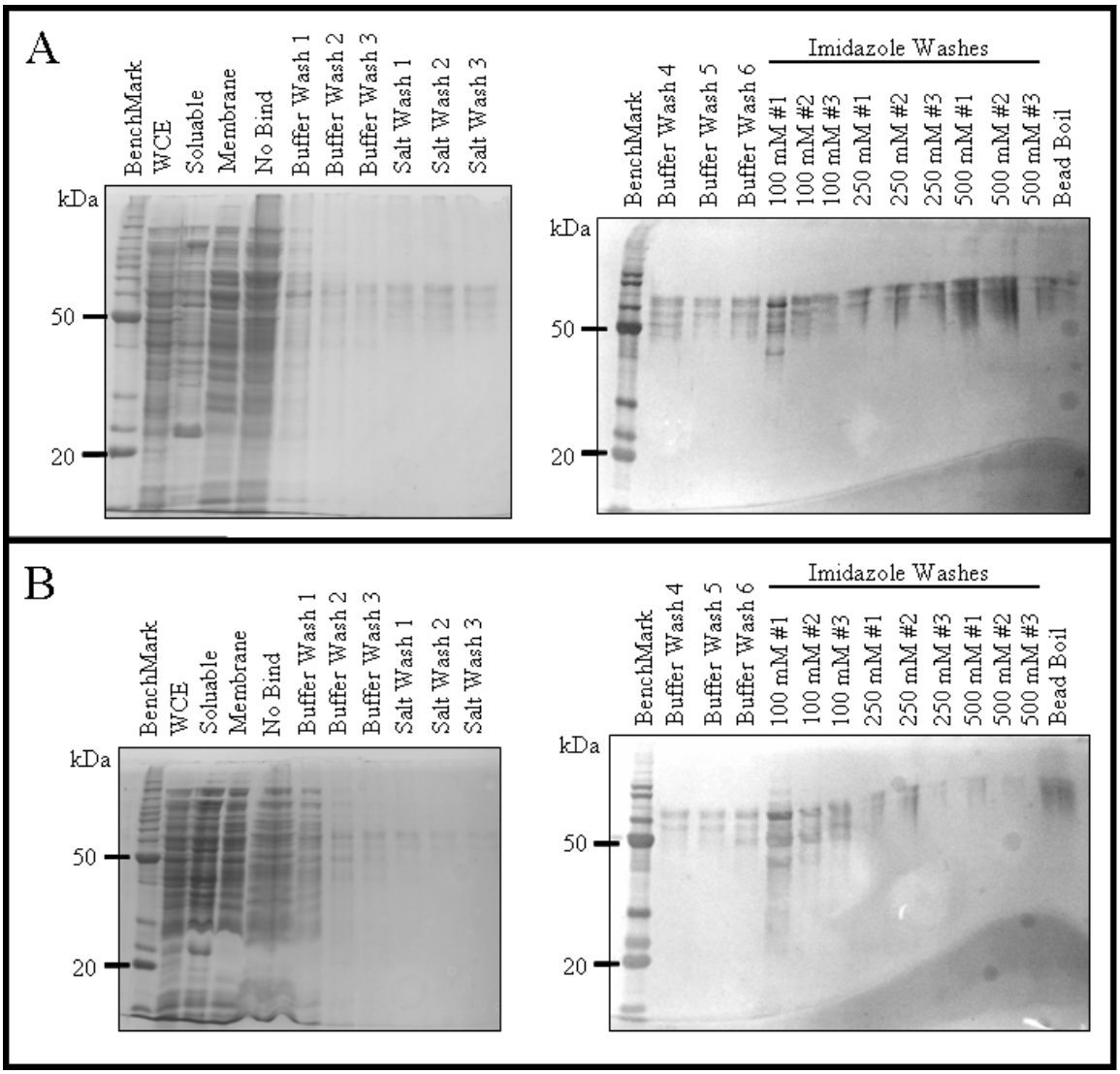

Figure 22

SDS-PAGE analysis of a Ni-NTA pull down assay using whole cell extract from a strain expressing His_8-FtsW. A) SDS-PAGE analysis of a Ni-NTA pull down assay performing using solubilized membrane fraction of AK4 (*his_8-ftsW*). The 12% gel containing early fractions and washes was stained with Coomassie blue (left) and 12% the gel containing late fractions was nickel-stained (right). B) SDS-PAGE analysis of a Ni-NTA pull down assay performing using solubilized membrane fraction of the wildtype strain M145. The 12% gel containing early fractions and washes was stained with Coomassie blue (left) and the 12% gel containing late fractions was nickel-stained (right). The predicted molecular weights for FtsI and FtsW are 69 kDa and 48 kDa, respectively.

cultures because this species does not sporulate in liquid. This may be another reason why additional proteins were not discovered. Regardless, this assay was performed to try a different method of studying protein-protein interactions without the use of α-FtsI antibodies, which bind nonspecifically to many proteins.

Another way to optimize detection of specific proteins in the Ni-NTA pull down assay would be to purify the α-FtsI antibodies. The first method used was discussed briefly while analyzing epitope-tagged FtsZ fusion proteins. The α-FtsZ antiserum was purified by incubating the antibodies with a membrane that contained whole cell extract from the *ftsZ*-null strain, partially removing nonspecific background antibodies from the serum (Figure 7A). The same approach was used while analyzing Ni-NTA pull down assays performed with the solubilized membrane fraction of AK4 (*his*$_8$-*ftsW*). The α-FtsI antiserum was first incubated with a blotted PVDF membrane that contained whole cell extract from the *ftsI*-null strain (Figure 23A). Background-subtracted antiserum was collected after incubation and used on another western blot membrane that contained fractions from the Ni-NTA pull down experiment (Figure 23B). His$_6$-`FtsI was overexpressed in *E. coli* and purified to use as a positive control (see below). It appeared that the α-FtsI antibodies used in this experiment are more pure, because background is lower (Compare Figures 19 and 21B); however, FtsI is still not visible in any lane on the membrane. These results confirm the idea that FtsI, and most likely FtsQ and FtsW, are not expressed at high enough levels when strains are grown in liquid culture.

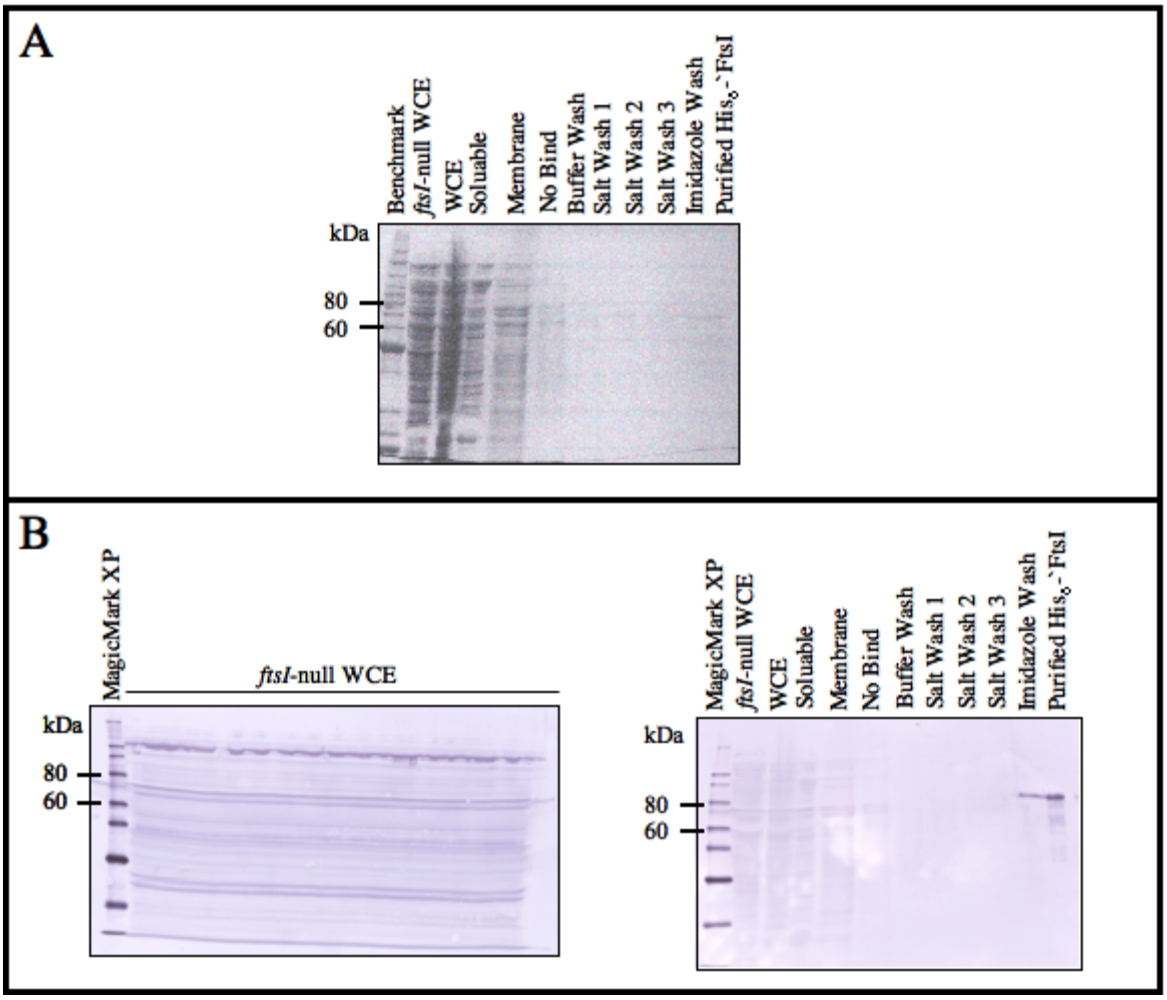

Figure 23

Western blot analysis of a pull down assay after partial purification of α-FtsI antibodies. A) Samples from an AK4 (His_8-FtsW) Ni-NTA pull down assay were fractionated on a 10% SDS-PAGE gel. This gel was stained with Coomassie blue. B) Partial purification of α-FtsI antibodies was carried out by incubating the antiserum with a membrane that contained whole cell extract (WCE) from the *ftsI*-null strain (left). Background-subtracted antiserum was then tested via western blot analysis of a duplicate 10% gel containing samples from an AK4 (His_8-FtsW) Ni-NTA pull down assay (right). The band seen in the "AK4 Imidazole Wash" lane is most likely protein from the purified His_6-`FtsI lane that had been misloaded. The predicted molecular weights of $His_6$-`FtsI and FtsI are 66.5 kDa and 69 kDa, respectively.

Attempts to Purify α-FtsI Antibodies

Affinity purification of α-FtsI antibodies was also attempted by covalently linking purified His_6-`FtsI to an activated medium, Affi-Gel 10 Gel (Bio-Rad), to create an affinity matrix. This gel couples to ligands via primary amines. $His_6$-`FtsI contains 118 amino acid residues with primary amine groups and therefore should bind efficiently to the resin. Affi-Gel 10 was chosen because it binds efficiently to proteins which possess an isoelectric point (pI) between 6.5 and 11. The estimated pI of His_6-`FtsI is 7.46. The following experiment was conducted by following a protocol provided by Dr. Nancy Kauffman (University of Pittsburgh: Pittsburgh, PA). His_6-FtsI was overexpressed in *E. coli* and purified using a Ni-NTA column. All fractions were analyzed via SDS-PAGE (Figure S9). Fractions containing purified His_6-`FtsI were pooled, concentrated, and incubated with the activated Affi-Gel 10 resin at 4°C overnight to allow binding between the protein and resin. The resin was washed the next day once with PBS and glycine and twice with TBST. All samples and washes were analyzed via SDS-PAGE to determine if the protein had bound to the resin. Based on this analysis, it appears that little His_6-`FtsI has bound to the Affi-Gel 10 (Figure S10). Multiple attempts were conducted using different preparatory methods. Dialysis of the protein solution was attempted to remove urea from the sample buffer, however the protein aggregated and precipitated rendering the sample unusable. Additional work needs to be carried out to optimize this method.

DISCUSSION

Cell division is a complex and essential process that has been studied in a number of prokaryotic microorganisms, most notably *E. coli* and *B. subtilis*; however, there is still much to be learned about this process. While many cell division genes have been identified, only three have a known function: FtsZ, FtsK, and FtsI. Studying interactions among cell division proteins may be helpful in identifying the remaining functions. Current experimental techniques being used to study these interactions are limited in *E. coli* and *B. subtilis* because most of the cell division genes are required for viability. Studying these putative protein-protein interactions in *S. coelicolor*, however, is advantageous for a number of reasons. The first is that these genes are dispensable. The second is that when cell division genes are deleted, the resulting strains usually have a specific phenotype, whether it be macroscopic, such as the "blue halo" phenotype of the *ftsZ*- and *ftsQ*-null strains, or microscopic, such as the medium-dependent phenotype associated with the *ftsL*-, *divIC*-, *ftsW*-, *ftsI*-null strains. These phenotypes and the dispensability of cell division genes can aid in the study of protein interactions because any allele can be introduced without loss of viability.

The purpose of this study was to initiate biochemical analyses of cell division protein interactions to complement genetic analyses that have been previously conducted. To that end, I have constructed epitope-tag cell division genes expressing proteins with His_8 or HA, utilizing cosmid derivatives that had been previously created, and have analyzed these fusion proteins using a Ni-NTA pull down assay to study protein-protein interactions in the divisome of this filamentous bacterium. I report here only my work

with completed strains. Additional constructs containing epitope-tagged genes expressing FtsL and DivIC are at various stages of completion.

Phase-contrast microscopy was used to determine whether the created epitope-tag fusion proteins being expressed in these strains were functional. The merodiploid strains AK1 (*ftsZ*$^{+}$/*his*$_{8}$-*ftsZ*) and AK18 (*his*$_{8}$-*ftsZ*/*his*$_{8}$-*ftsZ*) were the first to be studied. While the heterozygous strain AK1 was able to sporulate, the homozygous strain AK18 was unable. Instead of producing long chains of oval-shaped spores, this strain appeared to only produce long, undivided, coiled aerial hyphae (Figure 8). This phenotype is similar to the phenotype previous discovered in the *ftsZ*-null strain HU133 (McCormick *et al*., 1994). Although the aerial hyphae were found to be coiled in AK18, which indicates that this fusion protein is partially functional and can support division in the vegetative filaments.

On the other hand, it appears that all of the FtsQ fusion proteins created were entirely functional, because all of these strains were found to sporulate efficiently (Figure 11). Had these fusion proteins been nonfunctional, these newly created strains would have behaved like the *ftsQ*-null strain HU151. This strain was previous discovered to be unable to sporulate efficiently. In fact, this strain mainly produced long, undifferentiated aerial hyphae like those seen in the *ftsZ*-null strain (McCormick and Losick, 1996). These results indicate that FtsQ can be epitope-tagged at either the N-terminus or the C-terminus and still function properly.

At this time, it appears that epitope-tagged FtsW is not functional, while the FtsI fusion proteins may be partially functional. Most of these strains display a medium-dependent phenotype that is also observed in the *ftsW*- and *ftsI*-null strains (Bennett,

2007). When grown on R2YE, many of these strains were unable to sporulate. Short, thick aerial hyphae were observed (Figures 15 and 19). Interestingly, AK19 (*ftsI-ha*/*ftsI-ha*) was actually found to sporulate of R2YE while AK22 (*ftsI*$^{+}$/*ftsI-his*$_{8}$) was not. It would be expected that they would behave similarly if the 27 amino acid FRT scar peptide was inhibiting function. This result may indicate that the His_8 epitope tag, but not the HA epitope tag, was the cause of the defect. When grown on MM, some strains expressing the epitope-tagged FtsW and FtsI fusion proteins were found to sporulate efficiently, also just like the *ftsW*- and *ftsI*-null strains (Figures 15 and 19). This is consistent with the interpretation that this fusion protein is unable to support division, but the loss of function can be bypassed on MM. Another interesting phenotype was observed for the strains AK7 (*ftsW*$^{+}$/*ftsW-ha*) and AK23 (*ftsW-his*$_{8}$/*ftsW-his*$_{8}$), however. No spore chains were found to mature in either of these strains when grown on MM. It is apparent that epitope-tagging the C-terminus of FtsW creates a phenotype worse than not expressing any FtsW at all. These fusion proteins may be incorporating into the divisome and rendering it nonproductive.

To complete this analysis, all remaining merodiploid strains will have to be grown without antibiotic selection to allow intramolecular homologous recombination. These final strains will also have to be verified via Southern blot hybridization analysis to ensure that no rearrangement or deletion has occurred within the *dcw* gene cluster. Without this type of confirmation, it cannot be determined whether these phenotypes that were observed were caused by the fusion protein or by other unintended changes within the chromosome.

Many of the fusion proteins created in the study appeared to be nonfunctional (Table 13). This could be caused by the 81 bp *frt* scar site sequence that was included into each gene in addition to the epitope-tag sequence. If this additional scar sequence was removed from these genes, functional fusion proteins might be expressed. A method to remove this scar sequence and replace it with a smaller scar is being developed at this time (Figure 24). This technique would again rely on λ RED-mediated PCR targeting. A disruption cassette containing the spectinomycin-resistance gene has been previously created. *Sbf*I-specific restriction enzyme cut sites and homology to the *frt* scar sequence can be added to the ends of this disruption cassette utilizing the same PCR methods discussed above. The restriction enzyme *Sbf*I was chosen for this experiment because none of the mobilizable cosmids contain restriction sites specific for this enzyme. This cassette will be incorporated into mobilizable cosmids utilizing λ RED-mediated recombination in place of the *frt* site scar sequence. The disruption cassette can be removed by digesting the newly made cosmid with *Sbf*I. The cosmid will be ligated creating a 12 bp scar in place of the 81 bp *frt* site scar sequence. These cosmids will be incorporated into the *S. coelicolor* chromosome in the same manner discussed above. This method may be useful for quickly and efficiently creating functional fusion proteins using reagents at hand, which would require less time and resources than starting over.

These strains that express epitope-tagged cell division proteins were created so that protein interactions could be studied. To study these interactions, a Ni-NTA pull down assay was utilized. Using this technique, His_8-tagged proteins can be isolated by performing a pull down assay with Ni-NTA resin. Noninteracting proteins will be eluted by washing the resin with solubilization buffer and salt buffer. The His_8-tagged proteins,

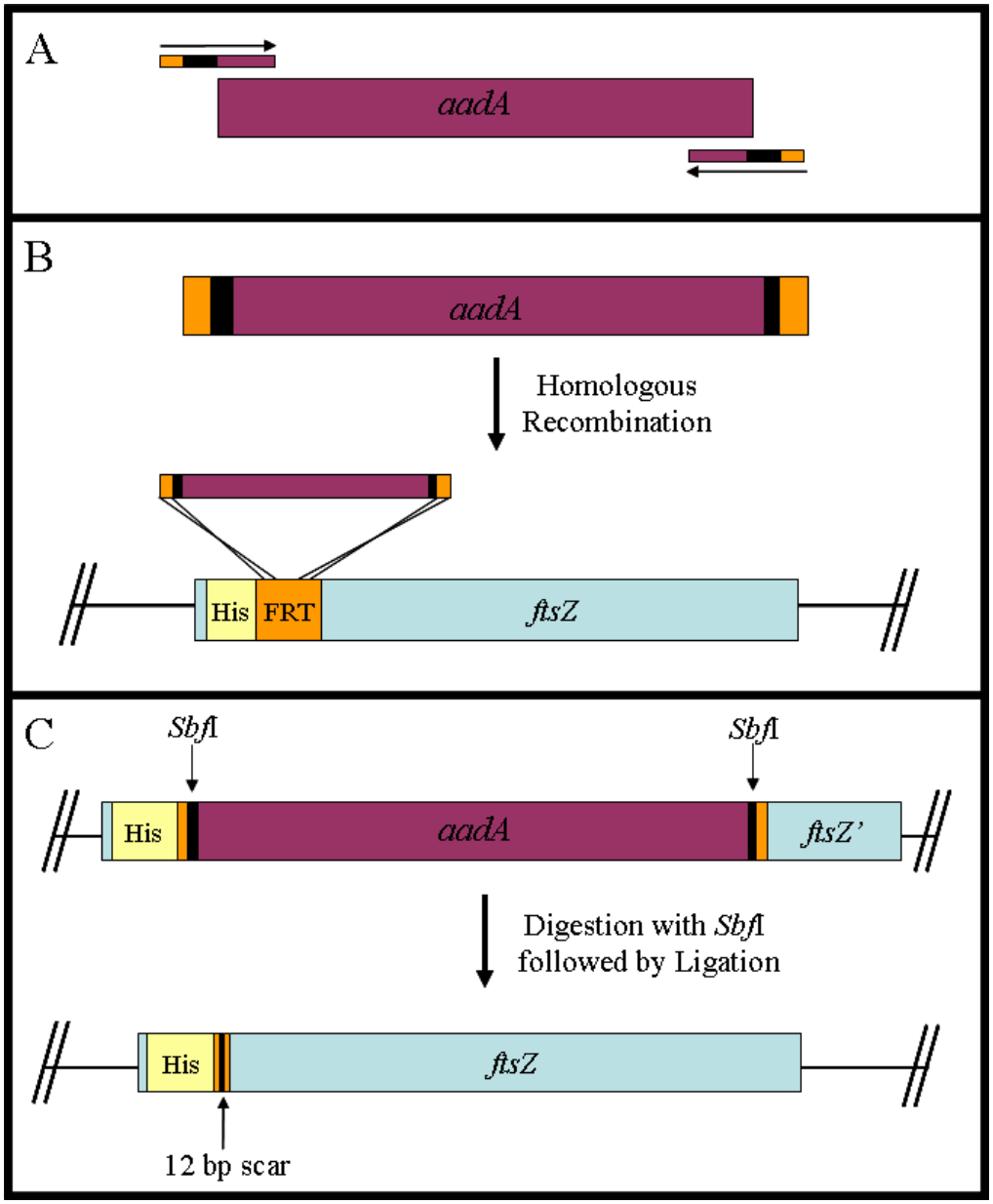

Figure 24

Strategy for replacing the 81 bp *frt* site scar sequence within mobilizable cosmids. This example explains how the 81 bp *frt* site scar sequence can be removed from the cosmid pAK1. A) *Sbf*I-specific restriction enzyme recognition sites and homology to the *frt* site scar sequence are added to an insertion-disruption cassette containing the spectinomycin-resistance gene (*aadA*) via PCR. B) The modified *aadA* cassette is incorporated into the *frt* scar sequence of pAK1 via λ RED-mediated recombination. C) The insertion-disruption cassette is removed by digesting the cosmid with *Sbf*I. The cosmid can be ligated resulting in a 12 bp scar sequence between the his_8 epitope tag sequence and the second codon of *ftsZ*. The orange boxes represent the *frt* site scar sequence and the black boxes represent *Sbf*I restriction sites.

as well as any interacting proteins, are eluted from the resin using imidazole buffer. Interacting proteins can be visualized via western blot analysis using α-FtsZ, α-FtsQ, α-FtsI, or α-HA antiserum. The strain AK1 (*ftsZ*$^{+}$/*his*$_{8}$-*ftsZ*) was utilized as a pilot for this experiment to demonstrate the method works. Because this strain expresses both wildtype FtsZ and also His$_{8}$-FtsZ, both proteins can be compared to ensure that only His$_{8}$-FtsZ binds the Ni-NTA resin. This protein was also chosen because it is abundant and cytoplasmic, which allows for easy isolation, and because highly specific polyclonal antibodies were available to visualize this protein via western blot analysis. When all fractions from the pull down were analyzed via western blot, His$_{8}$-FtsZ clearly bound to the resin while FtsZ did not (Figure 20). These results indicate that the Ni-NTA pull down assay could clearly be used to isolate His$_{8}$-tagged proteins.

The next step was to determine if interacting proteins could be co-eluted with His$_{8}$-tagged proteins. For this experiment, the strain AK4 (*his*$_{8}$- *ftsW*) was utilized to determine if FtsW and FtsI directly interact since this interaction had already been proposed in *E. coli* and *B. subtilis* by bacterial two-hybrid analysis (Di Lallo *et al*., 2003; Karimova *et al*., 2005; Daniel *et al*., 2006). Although it appears His$_{8}$-FtsW is not functional, this was the first strain to be created and membrane fractions were therefore used as a pilot to troubleshoot this method. Even though this fusion protein is unable to support division, it may still be stable enough to interact with some components of the divisome. In fact, it is possible that it may interact more efficiently and sequester some other component of the nonfunctional complex. The solubilized membrane fraction of an AK4 (*his*$_{8}$-*ftsW*) culture was utilized in these experiments to study the integral membrane cell division proteins instead of the soluble, cytoplasmic protein FtsZ. Fractions were

collected, fractionated by SDS-PAGE, and analyzed via western blot analysis using α-His and α-FtsI antibodies. Neither His_8-FtsW nor FtsI were visualized on these blots (Figure 21). This result could be attributed to the fact that His_8-FtsW is not functional or that the His_8 epitope tag is not stable *in vivo*. Even though the His_8-FtsZ fusion protein was found to be stable, this tag may be cleaved from FtsW. Another reason why these proteins are not visualized is probably because FtsW and FtsI are not expressed at a high enough level to be detected when cultures are grown in liquid. These proteins are not being highly expressed because *S. coelicolor* produces vegetative cross-walls, but does not divide extensively (sporulate) in liquid.

To overcome the problem of low expression levels, cultures can be grown on agar covered with cellophane. Cellophane is porous, allowing solutes and media to pass through, which permits vegetative filaments and aerial hyphae to grown on top of the cellophane. These cultures can be grown until just before sporulation, the stage at which the cell division proteins should be most highly expressed. The mycelia can be harvested and fractionated using the same method used for liquid grown cultures. Another way to overcome this problem would be to use a different species of *Streptomyces*, one that sporulates in liquid, to study protein-protein interactions. The genomes of *Streptomyces venezuelae* and *Streptomyces griseus* have been sequenced and these sequences should be released in the next year or two. This is important because the sequences of the cell division genes will be needed to created epitope-tagged fusion proteins using the method described in this thesis. Both of these species have also been found to sporulate in liquid, which will allow higher expression of cell division proteins in liquid-grown cultures. Also, the cosmid constructs, containing *S. coelicolor* genes, that were created in this

study might be usable to quickly introduce epitope-tagged genes into *S. venezuelae* and *S. griseus*, creating hybrid clones. This would allow for the study of *S. coelicolor* cell division proteins in a closely related organism that can express these proteins at a higher level when grown in liquid culture. All of these reasons make *S. venezuelae* and *S. griseus* ideal candidates for future studies.

Another problem encountered while conducting these pull down experiments is that the commercial α-His antibodies did not recognize any tested His_8-tagged protein. Since FtsZ was abundant enough in pull down fractions to be detected by α-FtsZ antibodies, this experiment was repeated using two different α-His antibodies (Qiagen and Santa Cruz). Both failed to visualize His_8-FtsZ (Figures S6 and S8). On the other hand, α-FtsI antibodies and α-FtsQ were found to have a high background and bound nonspecifically to many proteins (Figure 21 and data not shown). Because these antibodies could not be used at this time for western blot analysis, a different method of studying protein-protein interactions was explored. Instead of performing a western blot analysis on pull down fractions, fractions were analyzed by nickel-staining after fractionation by SDS-PAGE. This stain is more about ten times more sensitive than Coomassie stain and therefore may be able to detect low levels of cell division proteins. Neither His_8-FtsW nor FtsW-interacting proteins were visualized utilizing this method. This method may prove useful, however, in discovering novel interacting proteins if functional fusion proteins can be expressed at high enough levels to be visualized by nickel-staining.

To optimize detection of specific proteins in the Ni-NTA pull down assay, attempts were made to purify the α-FtsI polyclonal antibodies. The first method used to

partially purify these antibodies was to incubate them with a membrane that contained whole cell extract from the *ftsI*-null mutant. The background-subtracted antiserum was then used to analyze the blots from another pull down experiment using the strain AK4 (*his*$_8$-*ftsW*) (Figure 20). A band consistent with the molecular weight of FtsI was still not visualized, verifying that this protein is not expressed enough to be visualized using this method. However, the antibodies did specifically recognize purified His$_6$-`FtsI and not any other proteins. This method can therefore be used as a quick method for purifying α-FtsI antisera. Another method of purify antibodies would be to create an affinity column made from the protein of interest. An expression vector containing His$_6$-`FtsI was previously created and was used for this experiment. This protein was overexpressed, purified, concentrated, and incubated with Affi-Gel 10, an activated media that binds proteins via primary amines. Presently, very little His$_6$-`FtsI appeared to bind this matrix in my attempts; therefore, purification of antibodies has not yet been accomplished. More work needs to be done optimize this method.

In conclusion, the purpose of this thesis was to biochemically analyze putative cell division protein interactions in the filamentous organism *S. coelicolor* to complement and possibly expand upon prior genetic analyses. These analyses have required the creation of new tools, some of which have been previously created. I have completed the construction of *S. coelicolor* strains that express epitope-tagged FtsZ, FtsQ, FtsW, and FtsI. These strains have thus far been partially characterized. While some fusion proteins are functional, others appear to be either partially functional or completely nonfunctional. Other constructs to create strains expressing epitope-tagged FtsL and DivIC are currently in various stages of completion in *E. coli*. When completed, they will

be introduced into the chromosome of *S. coelicolor*. I have also utilized a Ni-NTA pull down assay to isolate His_8-tagged proteins and any interaction proteins. Visualization of FtsQ, FtsW, and FtsI via western blot analysis, however, has not been achieved at this time. A previous student was able to weakly detect FtsI via western blot (Yarnall, 2001); however this could not be duplicated in this study. Polyclonal antibodies against FtsQ and FtsI need to be affinity purified, while commercially available antibodies that reliably recognize the His_8 and HA epitope tags need to be obtained. The results of this thesis have set the stage to do a more thorough biochemical analysis of these putative protein interactions in the future.

REFRENCES

Addinall SG, Bi EF, Lutkenhaus J. FtsZ ring formation in *fts* mutants. J Bacteriol. 1996. **178**:877–3884.

Addinall SG, Cao C, Lutkenhaus J. FtsN, a late recruit to the septum in *Escherichia coli*. Mol Microbiol. 1997. **25**:303-309.

Addinall SG, Lutkenhaus, J. FtsZ-spirals and -arcs determine the shape of the invaginating septa in some mutants of *Escherichia coli*. Mol Microbiol. 1996. **22**:231–237.

Begg KJ, Hatfull GF, Donachie WD. Identification of new genes in a cell envelope-cell division gene cluster of *Escherichia coli*: cell division gene *ftsQ*. J Bacteriol. 1980. **144**:435-437.

Bennett JA. Molecular genetic analysis of division and development in *Streptomyces coelicolor*. Ph.D. Dissertation. Duquesne University: Pittsburgh, PA. 2006.

Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature. 2002. **417**:141-147.

Bernard CS, Sadasivam M, Shiomi D, Margolin W. An altered FtsA can compensate for the loss of essential cell division protein FtsN in *Escherichia coli*. Mol Microbiol. 2007. **64**:1289-1305.

Bernhardt TG, de Boer PA. The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. Mol Microbiol. 2003. **48**:1171-1182.

Bernhardt TG, de Boer PA. Screening for synthetic lethal mutants in *Escherichia coli* and identification of EnvC (YibP) as a periplasmic septal ring factor with murein hydrolase activity. Mol Microbiol. 2004. **52**:1255-1269.

Bi EF, Dai K, Subbarao S, Beall B, Lutkenhaus J. FtsZ and cell division. Res Microbiol. 1991. **142**:249-252.

Bi EF, Lutkenhaus J. FtsZ ring structure associated with division in *Escherichia coli*. Nature. 1991. **354**:161-164.

Bork P, Sander C, Valencia A. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc Natl Acad Sci USA. 1992. **89**:7290-7294.

Bidey PF. *ftsW* in *Streptomyces coelicolor* is not a filamentous temperature-sensitive wonder. B.S. Honors Thesis. Duquesne University: Pittsburgh, PA. 2004.

Boyle DS, Khattar MM, Addinall SG, Lutkenhaus J, Donachie WD. *ftsW* is an essential cell-division gene in *Escherichia coli*. Mol Microbiol. 1997. **24**:1263-1273.

Buddelmeijer N, Beckwith J. Assembly of cell division proteins at the *E. coli* cell center. Curr Opin Microbiol. 2002. **5**:553-557.

Buddelmeijer N, Beckwith J. A complex of the *Escherichia coli* cell division proteins FtsL, FtsB and FtsQ forms independently of its localization to the septal region. Mol Microbiol. 2004. **52**:1315-1327.

Buddelmeijer N, Judson N, Boyd D, Mekalanos JJ, Beckwith J. YgbQ, a cell division protein in *Escherichia coli* and *Vibrio cholerae*, localizes in codependent fashion with FtsL to the division site. Proc Natl Acad Sci USA. 2002. **99**:6316-6321.

Chen JC, Beckwith J. FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. Mol Microbiol. 2001. **42**:395-413.

Chen JC, Weiss DS, Ghigo JM, Beckwith J. Septal localization of FtsQ, an essential cell division protein in *Escherichia coli*. J Bacteriol. 1999. **181**:521-530.

Cherepanov PP, Wackernagel W. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene. 1995. **158**:9-14.

Chung KM, Hsu HH, Govindan S, Chang BY. Transcription regulation of *ezrA* and its effect on cell division of *Bacillus subtilis*. J Bacteriol. 2004. **186**:5926-5932.

Chung KM, Hsu HH, Yeh HY, Chang BY. Mechanism of regulation of prokaryotic tubulin-like GTPase FtsZ by membrane protein EzrA. J Biol Chem. 2007. **282**:14891-14897.

Corbin BD, Geissler B, Sadasivam M, Margolin W. Z-ring-independent interaction between a subdomain of FtsA and late septation proteins as revealed by a polar recruitment assay. J Bacteriol. 2004. **186**:7736-7744.

Corbin BD, Wang Y, Beuria TK, Margolin W. Interaction between cell division proteins FtsE and FtsZ. J Bacteriol. 2007. **189**:3026-3035.

D'Ulisse V, Fagioli M, Ghelardini P, Paolozzi L. Three functional subdomains of the *Escherichia coli* FtsQ protein are involved in its interaction with the other division proteins. Microbiology. 2007. **153**:124-138.

Dai K, Xu Y, Lutkenhaus J. Cloning and characterization of ftsN, an essential cell division gene in *Escherichia coli* isolated as a multicopy suppressor of ftsA12(Ts). J Bacteriol. 1993. **175**:3790-3797.

Daniel RA, Noirot-Gros MF, Noirot P, Errington J. Multiple interactions between the transmembrane division proteins of *Bacillus subtilis* and the role of FtsL instability in divisome assembly. Bacteriol. 2006. **188**:7396-7404.

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA. 2000. **97**:6640-6645.

de Boer PA, Crossley R, Rothfield L. The essential bacterial cell-division protein FtsZ is a GTPase. Nature. 1992. **359**:254-256.

Di Lallo G, Fagioli M, Barionovi D, Ghelardini P, Paolozzi L. Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation. Microbiology. 2003. **149**:3353-3359.

Draper GC, Gober JW. Bacterial chromosome segregation. Annu Rev Microbiol. 2002. **56**:567-97.

Draper GC, McLennan N, Begg K, Masters M, Donachie WD. Only the N-terminal domain of FtsK functions in cell division. J Bacteriol. 1998. **180**:4621-4627.

Errington J, Daniel RA, Scheffers DJ. Cytokinesis in bacteria. Microbiol Mol Biol Rev. 2003. **67**:52-65.

Geissler B, Elraheb D, Margolin W. A gain-of-function mutation in *ftsA* bypasses the requirement for the essential cell division gene *zipA* in *Escherichia coli*. Proc Natl Acad Sci USA. 2003. **100**:4197.

Geissler B, Margolin W. Evidence for functional overlap among multiple bacterial cell division proteins: compensating for the loss of FtsK. Mol Microbiol. 2005. **58**:596.

Ghigo JM, Beckwith J. Cell division in *Escherichia coli*: role of FtsL domains in septal localization, function, and oligomerization. J Bacteriol. 2000. **182**:116-129.

Goehring NW, Beckwith J. Diverse paths to midcell: assembly of the bacterial cell division machinery. Curr Biol. 2005. **15**:R514-R526.

Goehring NW, Gonzalez MD, Beckwith J. Premature targeting of cell division proteins to midcell reveals hierarchies of protein interactions involved in divisome assembly. Mol Microbiol. 2006. **61**:33-45.

Goehring NW, Gueiros-Filho F, Beckwith J. Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in *Escherichia coli*. Genes Dev. 2005. **19**:127-137.

Goehring NW, Robichon C, Beckwith J. Role for the nonessential N terminus of FtsN in divisome assembly. J Bacteriol. 2007. 189:646-649.

Gueiros-Filho FJ, Losick R. A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. Genes Dev. 2002. **16**:2544-2556.

Gust BT, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted *Steptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA. 2003. **100**:1541-1546.

Gust BT, Kieser T, and Chater K. REDIRECT technology: PCR-targeting system in *Streptomyces coelicolor*. John Innes Foundation: Norwich, England. 2002.

Haeusser DP, Schwartz RL, Smith AM, Oates ME, Levin PA. EzrA prevents aberrant cell division by modulating assembly of the cytoskeletal protein FtsZ. Mol Microbiol. 2004. **52**:801-814.

Hale CA, de Boer PA. Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. Cell. 1997. **88**:175-185.

Hale CA, de Boer PA. ZipA is required for recruitment of FtsK, FtsQ, FtsL, and FtsN to the septal ring in *Escherichia coli*. J Bacteriol. 2002. **184**:2552-2556.

Hale CA, de Boer PA. Recruitment of ZipA to the septal ring of *Escherichia coli* is dependent on FtsZ and independent of FtsA. J Bacteriol. 1999. **181:**167-176.

Hamoen LW, Meile JC, de Jong W, Noirot P, Errington J. SepF, a novel FtsZ-interacting protein required for a late step in cell division. Mol Microbiol. 2006. **59**:989-999.

Haney SA, Glasfeld E, Hale CA, Keeney D, He Z, de Boer PA. Genetic Analysis of the *Escherichia coli* FtsZ-ZipA Interaction in the Yeast Two-hybrid System: Characterization of FtsZ residues essential for the interactions with ZipA and with FtsA. J Biol Chem. 2001. **276**:11980-11987.

Hara H, Narita S, Karibian D, Park JT, Yamamoto Y, Nishimura Y. Identification and characterization of the *Escherichia coli envC* gene encoding a periplasmic coiled-coil protein with putative peptidase activity. FEMS Microbiol Lett. 2002. **212**:229-236.

Heidrich C, Templin MF, Ursinus A, Merdanovic M, Berger J, Schwarz H, de Pedro MA, Holtje JV. Involvement of N-acetylmuramyl-L-alanine amidases in cell separation and antibiotic-induced autolysis of *Escherichia coli*. Mol Microbiol. 2001. **41**:167-178.

Hirota Y, Ryter A, Jacob F. Thermosensitive mutants of *E. coli* affected in the processes of DNA synthesis and cellular division. Cold Spring Harb Symp Quant Biol. 1968. **33**:677–693.

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation: Norwich, England. 1985.

Ishikawa S, Kawai Y, Hiramatsu K, Kuwano M, Ogasawara N. A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in *Bacillus subtilis*. Mol Microbiol. 2006. **60**:1364-1380.

Jensen SO, Thompson LS, Harry EJ. Cell division in *Bacillus subtilis*: FtsZ and FtsA association is Z-ring independent, and FtsA is required for efficient midcell Z-Ring assembly. J Bacteriol. 2005. **187**:6536-6544.

Johnson JE, Lackner LL, Hale CA, de Boer PA. ZipA is required for targeting of DMinC/DicB, but not DMinC/MinD, complexes to septal ring assemblies in *Escherichia coli*. J Bacteriol. 2004. **186**:2418-2429.

Karimova G, Dautin N, Ladant D. Interaction network among *Escherichia coli* membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. J Bacteriol. 2005. **187**:2233-2243.

Kolisnychenko V, Plunkett G, Herring CD, Fehér T, Pósfai J, Blattner FR, Pósfai G. Engineering a reduced *Escherichia coli* genome. Genome Res. 2002. **12**:640-647.

Kieser T, Bibb MJ, Buttner M, Chater KF, Hopwood D. Practical *Streptomyces* genetics. John Innes Centre: Norwich, England. 2000.

Levin PA, Kurtser IG, Grossman AD. Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. Proc Natl Acad Sci USA. 1999. **96**:9642-9647.

Liu Z, Mukherjee A, Lutkenhaus J. Recruitment of ZipA to the division site by interaction with FtsZ. Mol Microbiol. 1999. **31:**1853-1861.

Löwe J, van den Ent F, Amos LA. Molecules of the bacterial cytoskeleton. Annu Rev Biophys Biomol Struct. 2004. **33:**177-198.

Ma X, Margolin W. Genetic and Functional Analyses of the Conserved C-Terminal Core Domain of *Escherichia coli* FtsZ. J Bacteriol. 1999. **181**:7531-7544.

MacNeil DJ, Occi JL, Gewain KM, MacNeil T, Gibbons PH, Ruby CL, Danis SJ. Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. Gene. 1992. **115**:119-125.

Margolin W. FtsZ and the division of prokaryotic cells and organelles. Nat Rev Mol Cell Biol. 2005. **6**:862-871.

Martin ME, Trimble MJ, Brun YV. Cell cycle-dependent abundance, stability and localization of FtsA and FtsQ in *Caulobacter crescentus*. Mol Microbiol. 2004. **54**:60-74.

Massey TH, Mercogliano CP, Yates J, Sherratt DJ, Lowe J. Double-stranded DNA translocation: structure and mechanism of hexameric FtsK. Mol Cell. 2006. **23**:457-469.

McCormick JR, Losick R. Cell division gene *ftsQ* is required for efficient sporulation but not growth and viability in *Streptomyces coelicolor* A3(2). J Bacteriol. 1996. **178**:5295-5301.

McCormick JR, Su EP, Driks A, Losick R. Growth and viability of *Streptomyces coelicolor* mutant for the cell division gene *ftsZ*. Mol Microbiol. 1994. **14**:243-254.

Morris JW. Genetic dissection of the cell division gene *ftsZ* in *S. coelicolor*. M.S. Thesis. Duquesne University: Pittsburgh, PA. 2002.

Pastoret S, Fraipont C, den Blaauwen T, Wolf B, Aarsman ME, Piette A, Thomas A, Brasseur R, Nguyen-Disteche M. Functional analysis of the cell division protein FtsW of *Escherichia coli*. J Bacteriol. 2004. **186**:8370-8379.

Pease PJ, Levy O, Cost GJ, Gore J, Ptacin JL, Sherratt D, Bustamante C, Cozzarelli NR. Sequence-directed DNA translocation by purified FtsK. Science. 2005. **307**:586-90.

Pichoff S, Lutkenhaus J. Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli*. EMBO J. 2002. **21**:685–693.

Pisabarro AG, Prats R, Vaquez D, Rodriguez-Tebar A. Activity of penicillin-binding protein 3 from *Escherichia coli*. J Bacteriol. 1986. **168**:199-206.

Quardokus EM, Din N, Brun YV. Cell cycle and positional constraints on FtsZ localization and the initiation of cell division in *Caulobacter crescentus*. Mol Microbiol. 2001. **39**:949-959.

Reddy M. Role of FtsEX in cell division of *Escherichia coli*: viability of *ftsEX* mutants is dependent on functional SufI or high osmotic strength. J Bacteriol. 2007. **189**:98-108.

Redenbach M, Kieser HM, Denapaite D, Eichner A, Cullum J, Kinashi H, Hopwood DA. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol Microbiol. 1996. **21**:7-96.

Romberg L, Levin PA. Assembly dynamics of the bacterial cell division protein FtsZ: poised at the edge of stability. Annu Rev Microbiol. 2003. **57:**25–154.

Saleh OA, Perals C, Barre FX, Allemand JF. Fast, DNA-sequence independent translocation by FtsK in a single-molecule experiment. EMBO J. 2004. **23**:2430-2439.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY. 1989.

Sanchez M, Valencia A, Ferrandiz MJ, Sandler C, Vicente M. Correlation between the structure and biochemical activities of FtsA, an essential cell division protein of the actin family. EMBO J. 1994. **13:**4919-4925.

Schmidt KL, Peterson ND, Kustusch RJ, Wissel MC, Graham B, Phillips GJ, Weiss DS. A predicted ABC transporter, FtsEX, is needed for cell division in *Escherichia coli*. J Bacteriol. 2004. **186**:785–793.

Schwedock J, McCormick JR, Angert ER, Nodwell JR, Losick R. Assembly of the cell division protein FtsZ into ladder-like structures in the aerial hyphae of *Streptomyces coelicolor*. Mol Microbiol. 1997. **25**:847-858.

Sivanathan V, Allen MD, de Bekker C, Baker R, Arciszewska LK, Freund SM, Bycroft M, Lowe J, Sherratt DJ. The FtsK gamma domain directs oriented DNA translocation by interacting with KOPS. Nat Struct Mol Biol. 2006. **13**:965-972.

Small E, Marrington R, Rodger A, Scott DJ, Sloan K, Roper D, Dafforn TR, Addinall SG. FtsZ polymer-bundling by the *Escherichia coli* ZapA orthologue, YgfE, involves a conformational change in bound GTP. J Mol Biol. 2007. **369**:210-221.

Spratt BG. Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. J Bacteriol. 1977. **131**:293-305.

Van De Putte P, Van Dillewijn, Roersch A. The selection of mutants of *Escherichia coli* with impaired cell division at elevated temperature. Mutat Res. 1964. **106**:121–128.

Vicente M, Rico AI. The order of the ring: assembly of *Escherichia coli* cell division components. Mol Microbiol. 2006. **61**:5-8.

Wang Y, Jones BD, Brun YV. A set of *ftsZ* mutants blocked at different stages of cell division in *Caulobacter*. Mol Microbiol. 2001. **40**:347-360.

Weaver D, Karoonuthaisiri N, Tsai HH, Huang CH, Ho ML, Gai S, Patel KG, Huang J, Cohen SN, Hopwood DA, Chen CW, Kao CM. Genome plasticity in *Streptomyces*: identification of 1 Mb TIRs in the *S. coelicolor* A3(2) chromosome. Mol Microbiol. 2004. **51**:1535-1550.

Weiss DS. Bacterial cell division and the septal ring. Mol Microbiol. 2004. **54**:588-597.

Yarnall JB. Characterization of the cell division gene *ftsI* in *Streptomyces coelicolor*. M.S. Thesis. Duquesne University: Pittsburgh, PA. 2001.

Yates J, Aroyo M, Sherratt DJ, Barre FX. Species specificity in the activation of Xer recombination at dif by FtsK. Mol Microbiol. 2003. **49**:241-249.

Virca GD, Northemann W, Shiels BR, Widera G, Broome S. Simplified northern blot hybridization using 5% sodium dodecyl sulfate. Biotechniques. 1990. **8**:370-371.

Yu XC, Tran AH, Sun Q, Margolin W. Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. J Bacteriol. 1998a. **180**:1296-1304.

Yu XC, Weihe EK, Margolin W. Role of the C terminus of FtsK in *Escherichia coli* chromosome segregation. J Bacteriol. 1998b. **180**:6424-6428.

APPENDIX

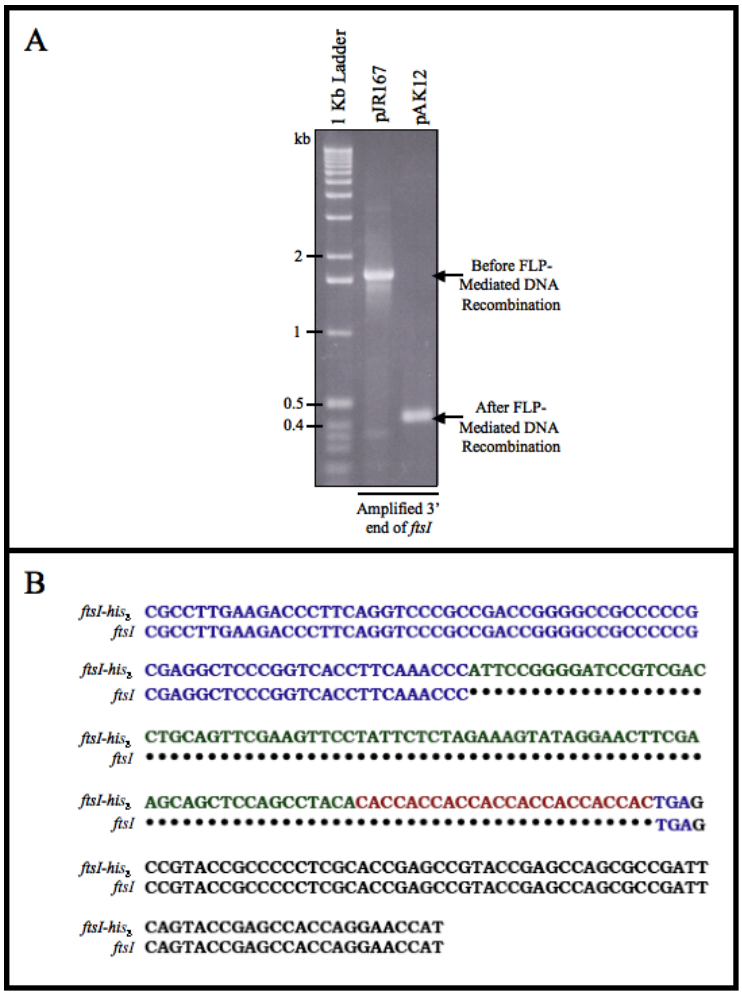

Figure S1

Verification of cosmid derivatives using PCR and sequencing analyses. A) After FLP-mediated DNA recombination, all cosmid derivatives were analyzed by PCR to ensure that recombination had occurred properly. In this example, the cosmid derivatives pJR167 (*ftsI-frt-aac(3)IV-oriT-frt-his*$_8$) and pAK12 (*ftsI-frt-his*$_8$) were analyzed. Before FLP-mediated DNA recombination, the expected amplicon size was 1689 bp. After FLP-mediated DNA recombination, the expected amplicon size was 388 bp. B) The 3' end of *ftsI* in the cosmid derivative pAK12 was sequenced and compared to the wildtype *ftsI* sequence to ensure recombination occurred without deletions or base substitutions. Blue bases indicate the *ftsI* sequence, green bases indicate the *frt* site scar sequence, red bases indicate the *his*$_8$ epitope-tag sequence, and black bases indicate the sequence downstream of *ftsI*. The symbol • indicates a gap in the wildtype sequence where the *his*$_8$ epitope-tag and the *frt* site scar sequences were inserted.

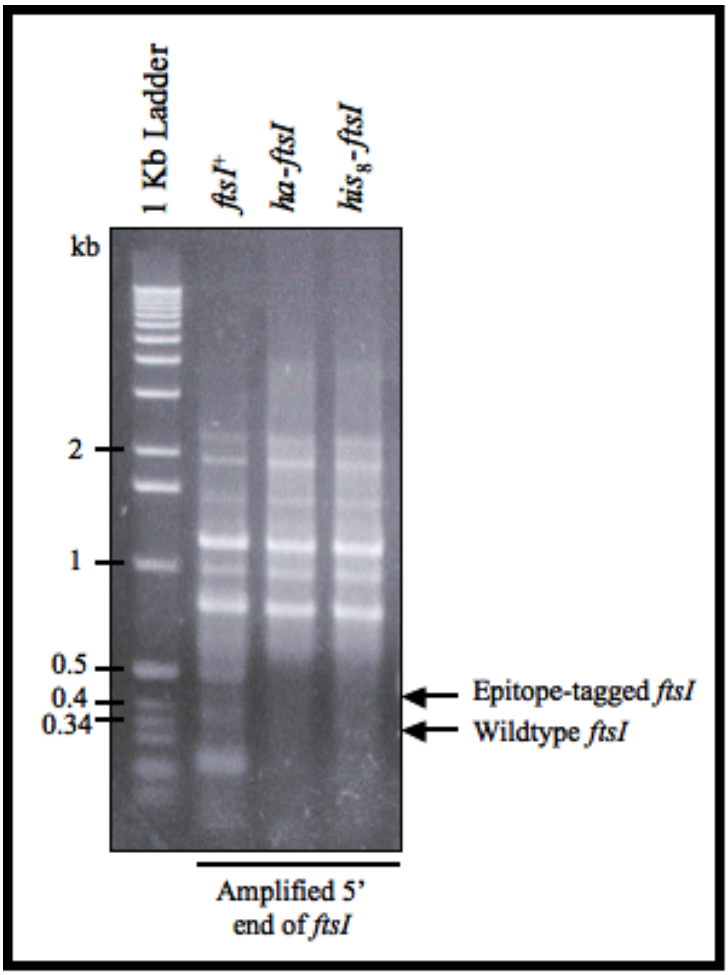

Figure S2

PCR analysis of cosmid derivatives using primers oI152 and oI167. The 5' end of *ftsI* was amplified in cosmids C69 (*ftsI*$^+$), pAK13 (*ha-ftsI*), and pAK14 (*his*$_8$*-ftsI*) to ensure that FLP-mediated DNA recombination had occurred properly. The expected amplicon size for *fstI*$^+$ was 319 bp and the expected amplicon sizes of *ha-ftsI* and *his*$_8$*-ftsI* were 427 and 424 bp, respectively. The primers oI152 and oI167 did not amplify the correct region of the cosmids, resulting in multiple background bands of incorrect size. Because of this, the 5' end of *ftsI* in the cosmid derivatives pAK13 and pAK14 could not be sequenced and these constructs could not be completed.

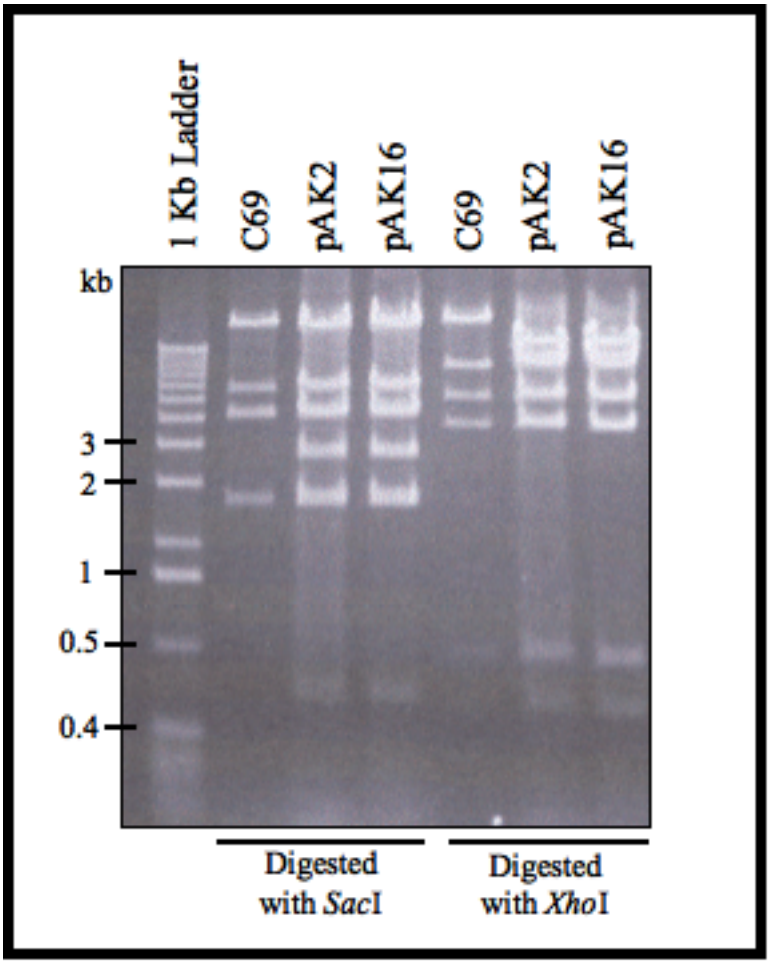

Figure S3

Restriction enzyme digestion analysis of mobilizable cosmid derivatives. All mobilizable cosmid derivatives were analyzed by restriction enzyme digestion to ensure that the ampicillin-resistance gene was replaced with the aparamycin-resistance gene and an *oriT* site. The insertion-disruption cassette used to make all cosmid derivatives mobilizable contains additional *Sac*I and *Xho*I restriction sites. Therefore, all mobilizable cosmids exhibit a unique banding pattern when compared to C69. In this example, the cosmid derivative pAK16 (pAK12 containing Δ*bla*::*aac(3)IV-oriT*) was compared to C69 and pAK2 (C69 containing Δ*bla*::*aac(3)IV-oriT*).

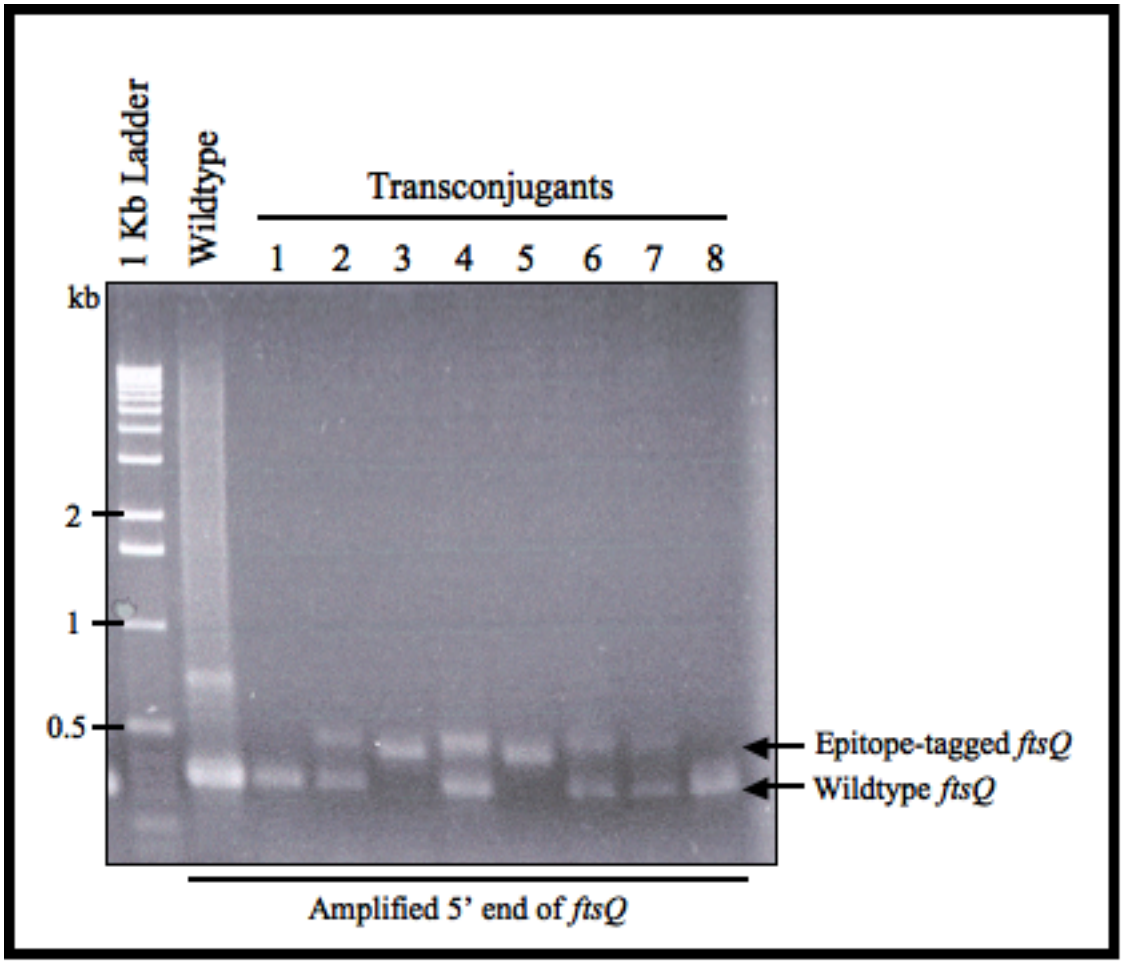

Figure S4

PCR analysis of *S. coelicolor* transconjugants expressing epitope-tagged cell division proteins. All mobilizable cosmid derivatives were incorporated into the chromosome of *S. coelicolor* via homologous recombination following conjugation. In this example, the cosmid derivative pAK6 (*ha-frt-ftsQ*) was transferred from *E. coli* to *S. coelicolor* resulting in merodiploid strains containing a duplicate division and cell wall gene cluster. All transconjugants were analyzed via PCR to determine their genotype. While many transconjugants were heterozygous (*ftsQ*$^{+}$/*ha-ftsQ*) (lanes 2, 4, 6 and 7), others underwent gene conversion and contained two wildtype copies of *ftsQ* (lanes 1 and 8) or two epitope-tagged copies of *ftsQ* (lanes 3 and 5).

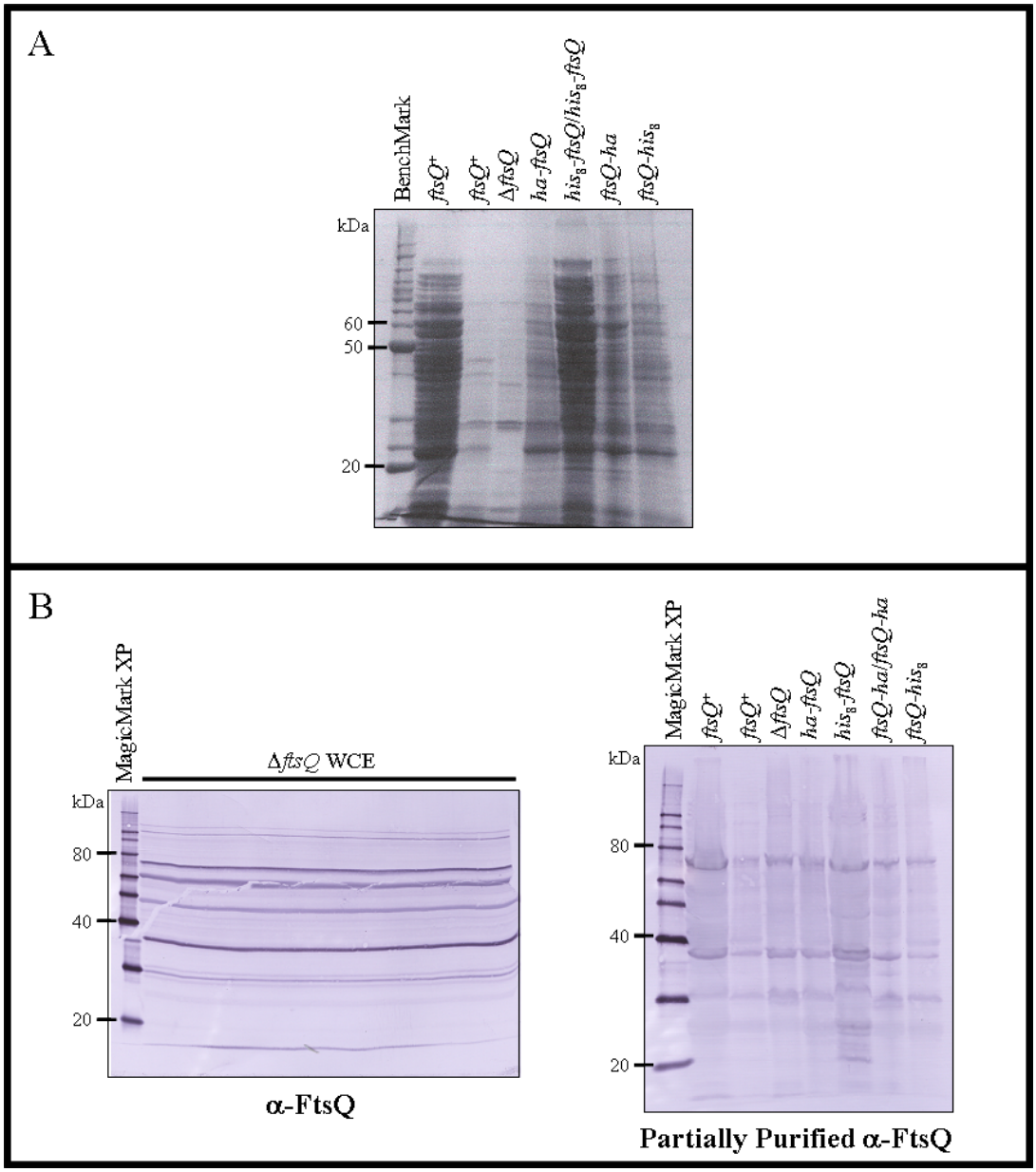

Figure S5

Attempt to visualize epitope-tagged FtsQ by western blot analysis. Whole cell extract (WCE) was obtained from the wildtype strain (*ftsQ*$^+$), the *ftsQ*-null strain (Δ*ftsQ*), AK13 (*ha-ftsQ*), AK16 (*his*$_8$-*ftsQ*/*his*$_8$-*ftsQ*), AK14 (*ftsQ-ha*), and AK15 (*ftsQ-his*$_8$). A) SDS-PAGE analysis containing samples of whole cell extract from each strain. The 12% gel was stained with Coomassie blue. B) Protein samples on a duplicate gel were electrotransferred onto a PVDF membrane and visualized using α-FtsQ antibodies (right). The antibodies were partially purified first by incubating them with a membrane that contained whole cell extract from the Δ*ftsQ* strain (left). Neither wildtype nor epitope-tagged FtsQ were visualized on this blot. The predicted molecular weight of FtsQ is 29 kDa.

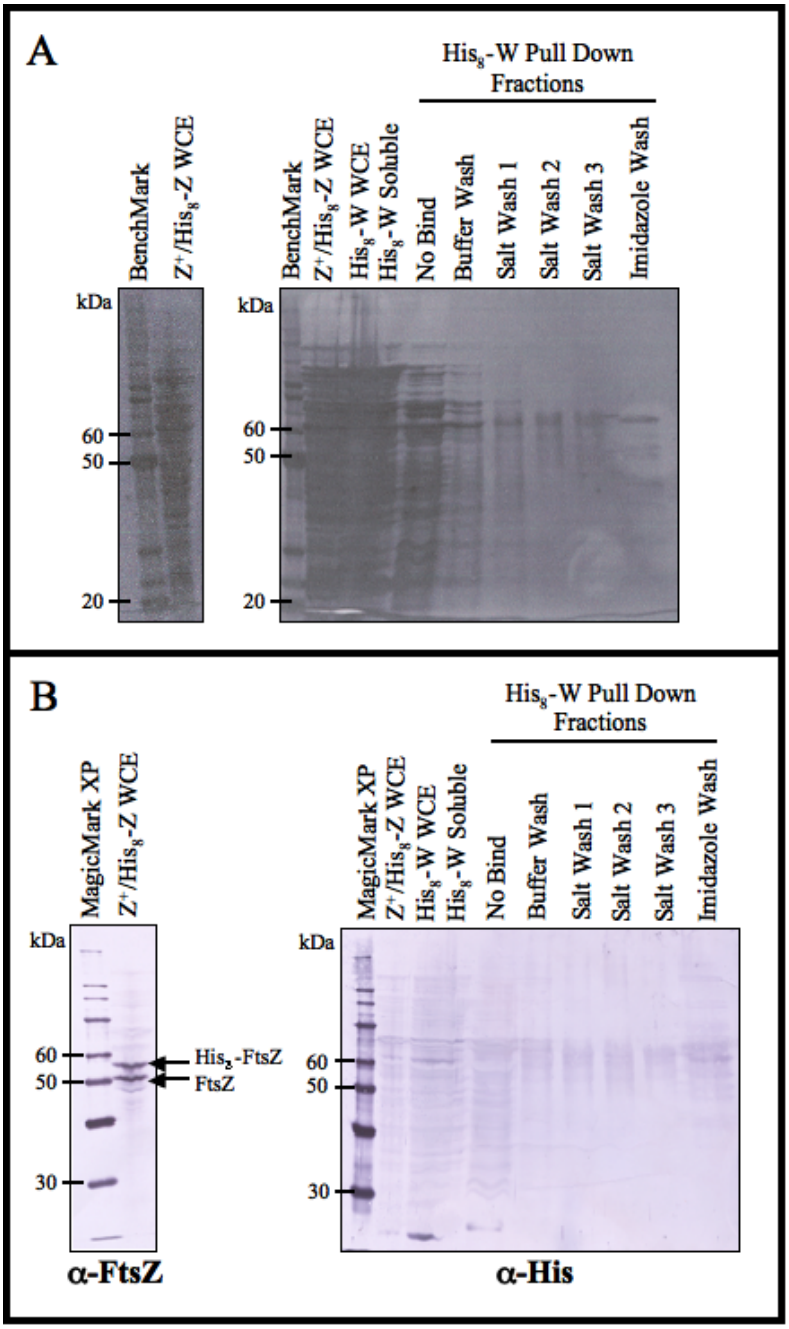

Figure S6

Attempt to visualize His_8-FtsW using α-His antibodies. An attempt to visualize His_8-FtsW was performed using α-His antibodies (Santa Cruz). A) An AK4 (His_8-FsW) pull down assay was performed and samples were fractionated on a 10% SDS-PAGE gel. The gel was stained with nickel. B) A duplicate gel was electrotransferred onto a PVDF membrane and analyzed using α-FtsZ antibodies (left) and α-His antibodies (right). Whole cell extract from the strain AK1 ($FtsZ^+$/His_8-FtsZ) was also loaded as a positive control. The α-His antibodies did not recognize any His_8-tagged protein.

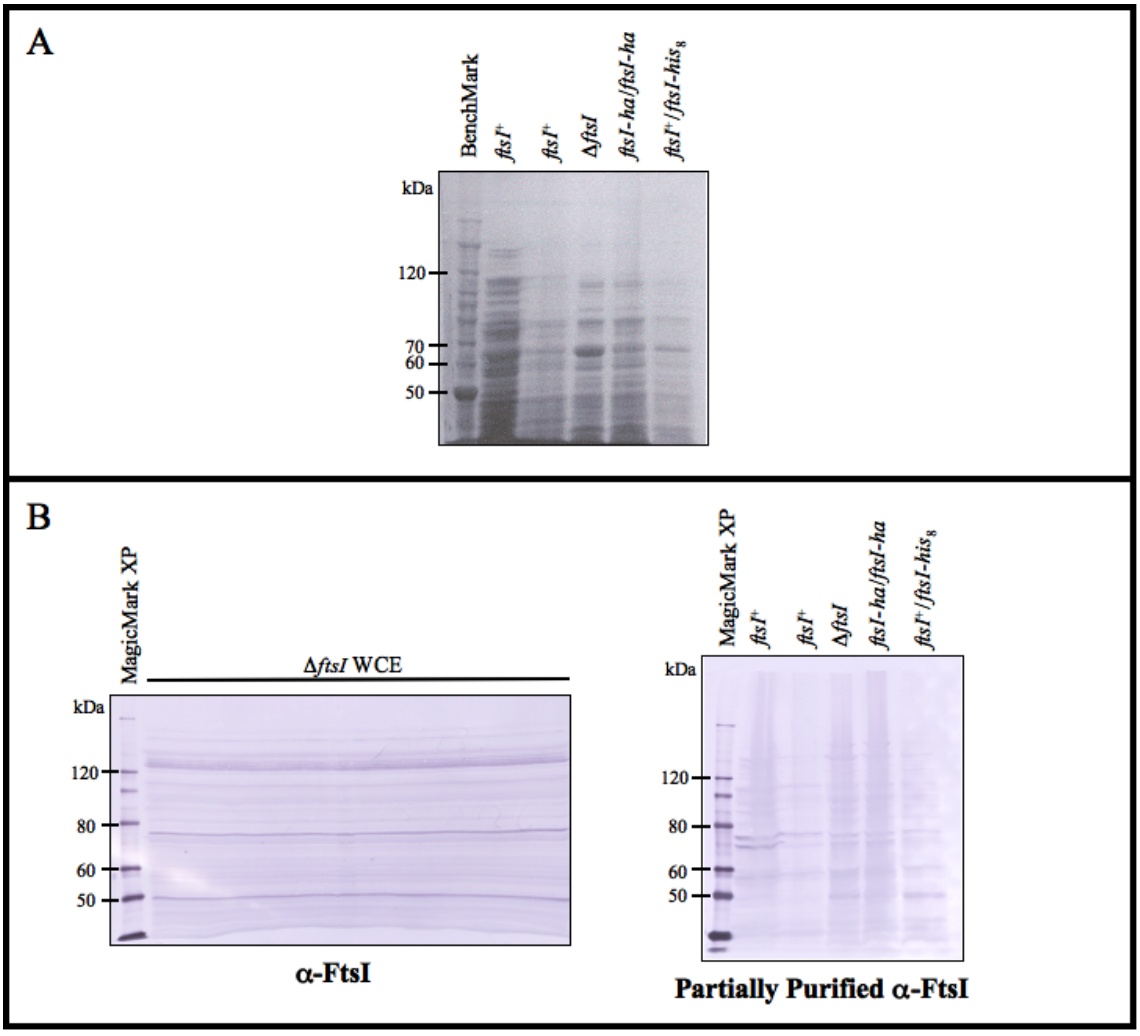

Figure S7

Attempt to visualize epitope-tagged FtsI by western blot analysis. Whole cell extract was obtained from the wildtype strain (*ftsI*$^+$), the *ftsI*-null strain (Δ*ftsI*), AK19 (*ftsI-ha*/*ftsI-ha*), and AK22 (*ftsI*$^+$/*ftsI-his*$_8$). A) SDS-PAGE analysis containing samples of whole cell extract from each strain. The 8% gel was stained with Coomassie blue. B) Protein samples on a duplicate gel were electrotransferred onto a PVDF membrane and visualized using α-FtsI antibodies (right). The antibodies were partially purified first by incubating them with a membrane that contained whole cell extract from the Δ*ftsI* strain (left). Neither wildtype nor epitope-tagged FtsI were visualized on this blot. The predicted molecular weight of FtsI is 69 kDa.

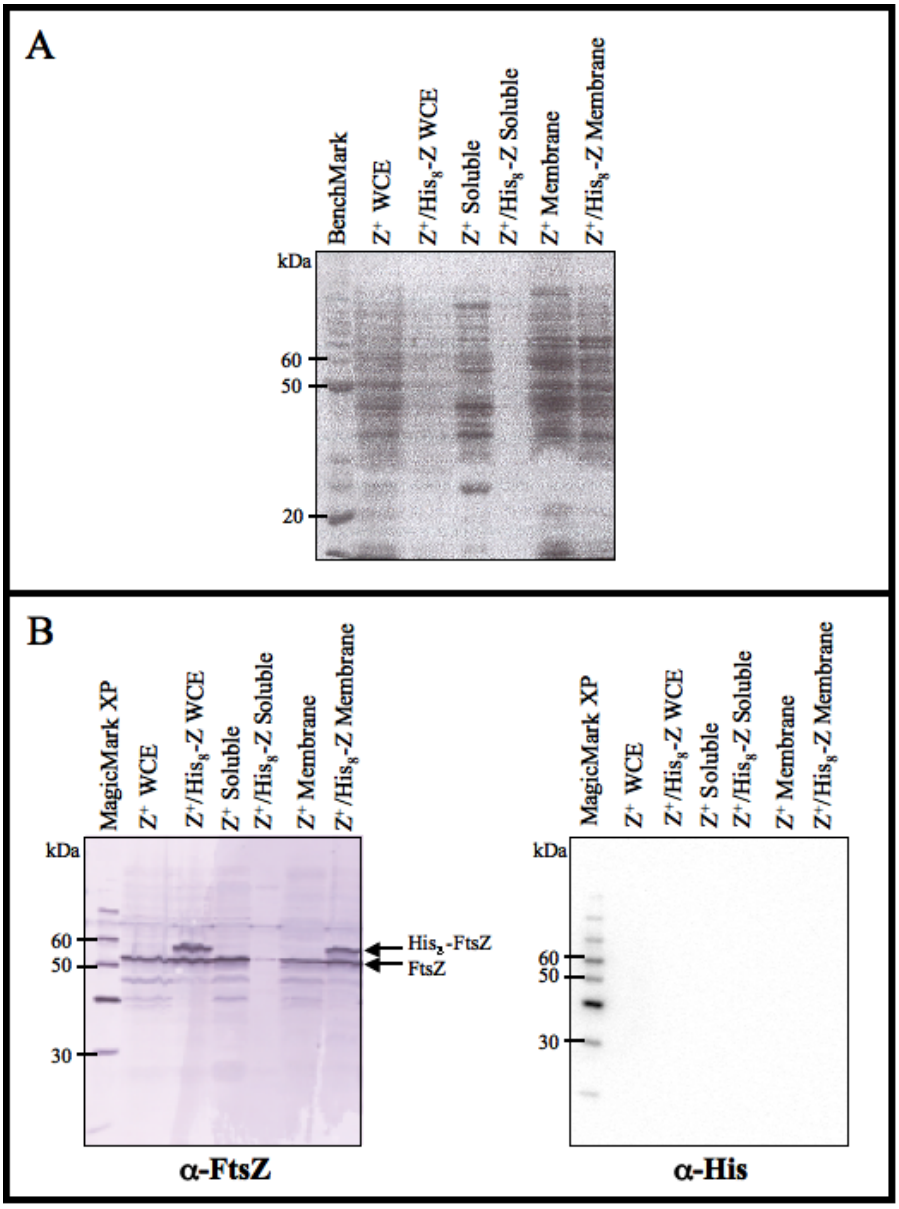

Figure S8

Attempt to visualize His_8-FtsZ using α-His antibodies. An attempt to visualize His_8-FtsZ was performed using α-His antibodies (Qiagen). A) Samples obtained from the wildtype strain M145 (Z^+) and the strain AK1 (Z^+/His_8-Z) were fractionated on a 10% SDS-PAGE gel. The gel was stained with Coomassie blue. B) A duplicate gel was electrotransferred onto a PVDF membrane and analyzed using α-FtsZ antibodies (left) and α-His antibodies (right). The α-His antibodies did not recognize any His_8-tagged protein. (WCE- whole cell extract)

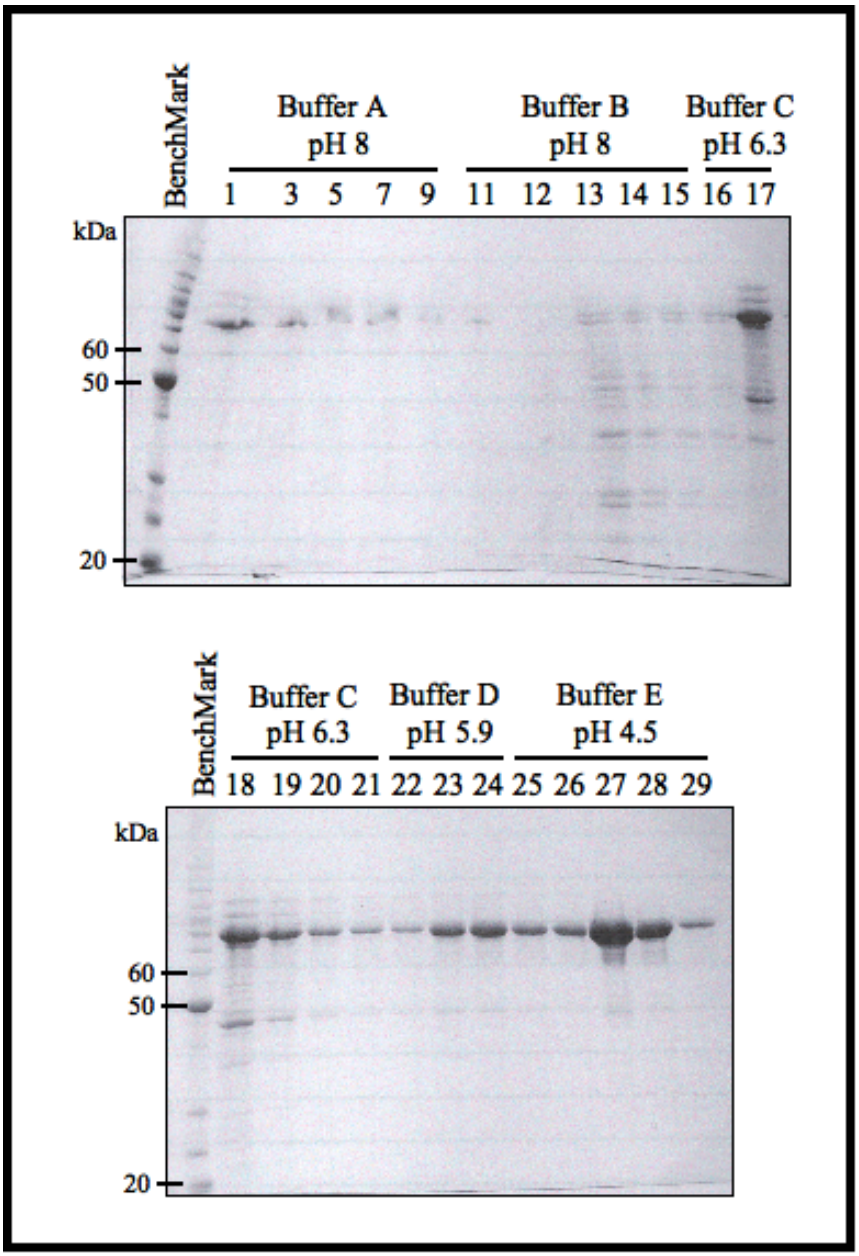

Figure S9

**Overexpression and purification of His_6-`FtsI.** $His_6$-`FtsI was overexpressed in *E. coli* and purified using a Ni-NTA column. The protein was eluted from the column with a decreasing pH gradient. Fractions were collected and analyzed on two10% SDS-PAGE gels. Both gels were stained with Coomassie blue. The Buffer D and Buffer E fractions were independently pooled and concentrated.

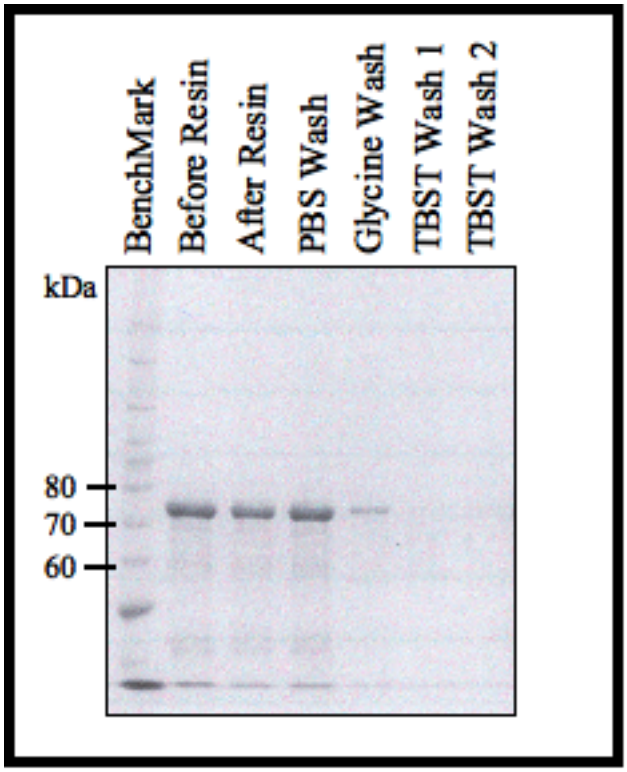

Figure S10

Attempt to create an affinity column to purify α-FtsI antibodies. Purified His_6-`FtsI was incubated with Affi-Gel 10 activated resin. The column was drained and washed with PBS, glycine, and TBST. Each sample was analyzed on a 10% SDS-PAGE gel. The gel was stained with Coomassie blue. Much of the purified $His_6$-`FtsI is visible after the incubation period with the resin and also after the first two washes. It appears that very little protein is actually coupling with the resin.